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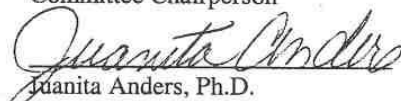
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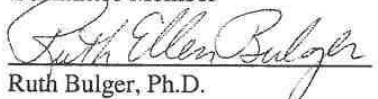
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Axons fail to regenerate after spinal cord injury (SCI) in adult mammals, leading to permanent loss of function. Following SCI, ensheathing cells promote recovery in animal models, whereas methylprednisolone promotes neurological recovery in humans. The aim of this research was to explore the effectiveness of ensheathing cells and methylprednisolone after acute SCI in the adult rat. Three studies were conducted to accomplish this goal. In the first study, a new method of purifying ensheathing cells was developed, resulting in a final population of ensheathing cells that were 93% pure. In the second study, the ability of a modified directed forepaw reaching (DFR) apparatus to accurately assess function of the corticospinal tract (CST) was examined. The data demonstrated that the modified apparatus prevented extinguishing of DFR behavior after SCI. In addition, the modified apparatus allowed for the collection of quantitative data to accurately assess CST function after bilateral, cervical spinal cord lesions. In the third study, the effectiveness of combining ensheathing cells and methylprednisolone after SCI was investigated. After lesioning the CST in adult rats, a purified population of ensheathing cells was transplanted into the lesion, and methylprednisolone was administered for 24 hours. At six weeks post injury, functional recovery was assessed by measuring successful DFR performance. Axonal regeneration was analyzed by counting the number of anterogradely labeled CST axons caudal to the lesion. Lesioned control rats, receiving either no treatment or vehicle, had abortive axonal regrowth (1 mm) and poor DFR success (38% and 42%, respectively). Compared to controls, rats treated with methylprednisolone for 24 hours had significantly more axons at 7 mm caudal to the lesion, and DFR performance was significantly improved (57%). Rats that received ensheathing cells with methylprednisolone had significantly more regrowing axons than all other lesioned rats up to 13 mm caudal to the lesion. Successful DFR performance was significantly higher in rats with ensheathing cell transplants, both without (72%) and with (78%) methylprednisolone, compared to other lesioned rats. These data confirm previous reports that ensheathing cells promote axonal regeneration and functional recovery after spinal cord lesions in a rat model. In addition, this research provides new evidence that, when used in combination, methylprednisolone and ensheathing cells improve axonal regrowth up to 13 mm caudal to the lesion.

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ABSTRACT

REGENERATION OF THE ADULT RAT SPINAL CORD IN RESPONSE TO ENSHEATHING CELLS AND METHYLPREDNISOLONE

Holly H. Nash

Directed by Juanita J. Anders, Ph.D., Associate Professor of Anatomy,
Physiology, and Genetics, and Neuroscience

Axons fail to regenerate after spinal cord injury (SCI) in adult mammals, leading to permanent loss of function. Following SCI, ensheathing cells promote recovery in animal models, whereas methylprednisolone promotes neurological recovery in humans. The aim of this research was to explore the effectiveness of ensheathing cells and methylprednisolone after acute SCI in the adult rat. Three studies were conducted to accomplish this goal. In the first study, a new method of purifying ensheathing cells was developed, resulting in a final population of ensheathing cells that were 93% pure. In the second study, the ability of a modified directed forepaw reaching (DFR) apparatus to accurately assess function of the corticospinal tract (CST) was examined. The data demonstrated that the modified apparatus prevented extinguishing of DFR behavior after SCI. In addition, the modified apparatus allowed for the collection of quantitative data to accurately assess CST function after bilateral, cervical spinal cord lesions. In the third study, the effectiveness of combining ensheathing cells and methylprednisolone after SCI was investigated. After lesioning the CST in adult

rats, a purified population of ensheathing cells was transplanted into the lesion, and methylprednisolone was administered for 24 hours. At six weeks post injury, functional recovery was assessed by measuring successful DFR performance. Axonal regeneration was analyzed by counting the number of anterogradely labeled CST axons caudal to the lesion. Lesioned control rats, receiving either no treatment or vehicle, had abortive axonal regrowth (1 mm) and poor DFR success (38% and 42%, respectively). Compared to controls, rats treated with methylprednisolone for 24 hours had significantly more axons at 7 mm caudal to the lesion, and DFR performance was significantly improved (57%). Rats that received ensheathing cells with methylprednisolone had significantly more regrowing axons than all other lesioned rats up to 13 mm caudal to the lesion. Successful DFR performance was significantly higher in rats with ensheathing cell transplants, both without (72%) and with (78%) methylprednisolone, compared to other lesioned rats. These data confirm previous reports that ensheathing cells promote axonal regeneration and functional recovery after spinal cord lesions in a rat model. In addition, this research provides new evidence that, when used in combination, methylprednisolone and ensheathing cells improve axonal regrowth up to 13 mm caudal to the lesion.

**REGENERATION OF THE ADULT RAT SPINAL CORD IN RESPONSE TO
ENSHEATHING CELLS AND METHYLPREDNISOLONE**

By

Holly H. Nash

Dissertation submitted to the faculty of the

Program in Neuroscience of the

Uniformed Service University of the Health Sciences

In partial fulfillment of the requirements for the degree of

Doctor of Philosophy 2002

DEDICATION

I dedicate this body of work:

To Cris

For all your love, support, encouragement, and strength

and

To Grandma

For everything

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I am thankful beyond words to my mentor, advisor, advocate and friend, Dr. Juanita J. Anders, for helping me grow as a scientist and a person. As an outstanding role model, she has changed my life in countless positive ways, and I am deeply grateful.

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Appendix 1

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LIST OF ABBREVIATIONS

A/P	anterior/posterior
BDNF	brain-derived neurotrophic factor
BDT	biotin dextran tetramethylrhodamine
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CST	corticospinal tract
DFR	directed forepaw reaching
DMEM	Dulbecco's minimum essential media
EC	ensheathing cells
FBS	fetal bovine serum
GDNF	glial cell-line derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GR	glucocorticoid receptor
HBSS	Hank's balanced salt solution
HSP 90	heat shock protein 90
M/L	medial/lateral
MP	methylprednisolone
NF- κ B	nuclear factor kappa-B
NGF	nerve growth factor

NT-3	neurotrophin-3
PNS	peripheral nervous system
PSB	phosphate buffered saline
RGC	retinal ganglion cells
SCI	spinal cord injury
TNF- α	tumor necrosis factor-alpha

CHAPTER 1

Introduction and Background

One having a dislocation in a vertebra of his neck while he is unconscious of his two legs and his two arms, and his urine dribbles. An ailment not to be treated.

Edwin Smith Papyrus, 1700 BC (Breasted, 1930)

The frog instantly dies when the spinal cord is pierced; and previous to this it lived without head, without heart or bowels or intestines or skin; and here therefore it would seem lies the foundation of movement and life.

Leonardo daVinci, 1452-1519 (Yashon, 1986)

The Spinal Cord

The nervous system is divided into two parts, the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS is composed of the brain and spinal cord, while the PNS is made up of the ganglia and nerves that lie outside the CNS. The brain is the most rostral portion of the CNS, while the spinal cord is the most caudal portion, extending from the base of the skull to the first lumbar vertebra in adult human beings, and is about 18 inches long. The spinal cord receives sensory information from the internal organs and has groups of neurons, called ganglia, that control many visceral functions. The spinal cord

also receives sensory information from the skin, muscles, and joints of the limbs and trunk, which is carried to the brain via ascending tracts. In addition, the spinal cord contains lower motor neurons, which receive information from upper motor neurons in the brain via descending tracts, responsible for voluntary and reflex motor movements (Kelly and Dodd, 1991).

The spinal cord is divided into four major regions: cervical, thoracic, lumbar and sacral, each of which contains numerous segments. Each segment has a corresponding pair of spinal nerves, and in human beings there are 31 pairs of spinal nerves: eight cervical, twelve thoracic, five lumbar, five sacral and one coccygeal. Spinal nerves are composed of peripheral nerves formed by the joining of dorsal roots, which carry sensory information into the spinal cord, and ventral roots, which carry motor information out of the spinal cord. Generally speaking, cervical spinal nerves innervate the neck and upper extremities, thoracic spinal nerves chiefly innervate trunk musculature, and lumbar and sacral spinal nerves innervate the lower extremities and genitalia (Kelly, 1991).

The integration of sensory and motor information is a complex process. One of the simplest parts of the CNS to understand and study is the corticospinal tract (CST). Specific regions of the motor cortex in the brain regulate the activity of specific motor groups in the periphery. Pyramidal neurons reside within layer V of the primary motor cortex, and it is the axons of these neurons that primarily compose the CST, although axons from the premotor, supplementary motor, and primary somatosensory cortices also contribute to this tract. In the brain, CST axons descend from these cortices, through the internal capsule, the cerebral

peduncle, and the medullary pyramids, where the majority of the CST crosses to form the contralateral CST. In human beings, the lateral CST courses in the lateral funiculus. The axons of the CST terminate in the spinal cord on alpha motor neurons and on interneurons that synapse on alpha motor neurons. The lower motor neurons regulated directly by the CST innervate distal muscles that are important for fine and skilled motor movements of the hand (Kelly, 1991).

Spinal Cord Injury

It is believed that there are currently 183,000 – 230,000 people in the United States living with a spinal cord injury (SCI), and that this number increases by 11,000 annually. However, since there has not been an overall SCI incidence study in the United States since the 1970's, the incidence rates are only estimates. The National Spinal Cord Injury Database, established in 1973, captures data from an estimated 13% of new SCI patients in the United States. Information collected in this database indicates that 82% of people with SCI are male, and 55% of SCI occur in persons 16-30 years old. While these statistics have not changed much over the last three decades, the racial distribution of SCI has changed dramatically. Between 1973 and 1978, Caucasians made up 77.5% of the SCI population, while African-Americans comprised only 13.5%. However, since 1990, the percentage of Caucasians injured has dropped to 59.1% and the percentage of African-Americans has risen to 27.6%. While motor vehicle accidents still account for the majority of SCI (38.5%), and sporting accidents are still a significant cause of SCI (7.2%), the proportion of SCI resulting from these two etiologies has been declining since 1973. The

proportion of SCI resulting from acts of violence (24.5%, primarily gunshot wounds) and falls (21.8%), however, are on the rise. Overall, 51.6% of persons have an injury located in the cervical region of the spinal cord that results in tetraplegia (formally called quadriplegia), or paralysis of the upper and lower extremities; the remaining 46.3% have injuries below the middle thoracic level that result in paraplegia (Center, 2001), or paralysis of the lower extremities.

In general, a SCI may cause, below the level of the lesion, a loss of sensory and/or motor function, low blood pressure, inability to regulate blood pressure effectively, reduced control of body temperature, inability to sweat, and chronic pain. It is the level of the SCI and the lesion position within the spinal cord that determines specifically what parts of the body are impaired. In addition to the symptoms listed above, injuries at the cervical (C) C1 to C4 levels can result in the loss of many involuntary functions including the ability to breathe, necessitating the use of breathing aids such as diaphragmatic pacemakers or mechanical ventilators. Paralysis in these patients usually extends from the neck down. A person injured between C5 and C7 has varying degrees of control of the shoulder, forearm, wrist and hand; but generally excludes dexterity of the fingers. Thoracic injuries result in varying degrees of trunk control, however, function of the lower limbs is abolished. Lumbar and sacral injuries produce decreased control of the hip and leg flexors, respectively (Center, 2000).

Early treatment and improved hospital care have increased survival and recovery rates. The cost of long-term, specialized care for paralyzed patients has also increased. The average cost for the first year after SCI is \$168,627 -

\$572,178, and ranges from \$11,817 - \$102,491 for each subsequent year, depending on the level of the injury. The estimated lifetime cost for one person who suffers a C1-C4 SCI at 25 years of age, is \$2,185,667. While these costs are staggering, the personal costs to the patients and their families are beyond calculation; planned education, career, marriage, and independence are interrupted and often never regained. Since the early 1990's, the use of methylprednisolone immediately after injury has resulted in enhanced neurological recovery (Bracken et al., 1990; Bracken et al., 1997), which can improve quality of life. Currently, however, there is no cure for SCI. Therefore, research efforts yielding information that, in the future, might reduce costly hospital and post-injury rehabilitation, expedite recovery time, reduce emotional suffering, and improve the level of post-injury function, are worthy and highly desired. Herein is such a study.

Spinal Cord Injury Research

Animal and injury models

The severe disabilities resulting from SCI have lead to the search for a cure. SCI models have been developed to allow an experimental approach to understanding the pathology of human SCI and the development of potential therapeutic interventions. The anatomical organization of the rat spinal cord is not identical to that of a human being. For example, the rat spinal cord, which is about 6 inches long, has eight pairs of cervical spinal nerves, thirteen thoracic, six lumbar, four sacral, and three coccygeal spinal nerves (Zeman and Innes,

1963). Another example is the location of the CST, which runs in the lateral funiculus in human beings, but is found in the ventral portion of the dorsal funiculus in rats. These differences, however, do not hinder the rat from being a useful model for SCI. In fact, several laboratories have demonstrated reproducible SCI models in the rat, including crush injury models (Wrathall, 1992b), partial transection models (Bregman et al., 1998), and complete transection models (Ramon-Cueto et al., 1998). Crush and transection models cause damage to numerous spinal cord tracts, and result in numerous functional deficits. Using these models, the effects of therapeutics on a single spinal cord tract are difficult to discern. Therefore, in order to examine the effects of therapeutics on a single spinal cord system, partial transection is the best injury model to utilize.

In addition to the many types of injury models that have been explored, numerous spinal cord tracts have been utilized to examine various aspects of injury and repair. One spinal cord tract that is commonly used to examine axonal regeneration and functional recovery is the CST. The CST is a good system to examine axonal regeneration and functional recovery for a number of reasons. The CST, as mentioned above, is one of the simplest motor systems of the CNS. Therefore, there is a direct correlation between CST injury and functional deficit. In addition, the anatomical location of the CST in the rat allows for lesioning of the CST without damaging any additional motor systems. The function of the CST, in both human beings and rats, is to control fine and skilled movements.

Lesioning of the cervical CST in rats produces discrete deficits in fine and skilled movements of the forepaws.

Numerous tests exist that examine general motor function, including the open field test (Wrathall, 1992a; Basso et al., 1995; von Euler et al., 1996) and the incline plane test (Rivlin and Tator, 1977; Gale et al., 1985; Kerasidis et al., 1987). While these tests can be used to describe an animal's general motor performance, they are not able to provide information about improvements in function of a specific motor tract (Kunkel-Bagden et al., 1993). Since damage to the CST does not result in gross motor impairments in the rat, tests such as the open field and inclined plane tests are not adequate to assess injury and repair of this system. The sticker removal test has been utilized to examine forelimb impairment (Schrimsher and Reier, 1992; Diener and Bregman, 1998a). However, while the sticker removal test can be employed to examine forelimb movement, it is not sufficient for analyzing varying degrees of CST impairment and improvement. In this test, a sticker is placed on the bridge of a rat's nose. Removal of the sticker requires full range of motion of the forelimbs and is facilitated if some grasping ability is intact. A rat's behavior when performing this task is rated on a scale from 0 to 6. A rat can score up to a 4 on this scale without ever attempting to grasp the sticker, and can score a 5 if the sticker is removed after numerous attempts without a strong grasp. If the sticker is tightly grasped and quickly removed, then a score of 6 is given. As five of the seven scoring measures used to assess this task are based on forelimb movement, not grasping ability, the amount of quantitative data that can be obtained from

utilizing this test to examine the range of CST integrity is limited. Therefore, sticker removal is not the ideal test for examining CST integrity of the forepaws.

The directed forepaw reaching (DFR) task, however, can be used to examine the integrity of the CST in rodents after cervical lesions resulting in quantitative data that can be used to determine not only whether the CST is damaged, but can also indicate the degree of damage or repair (Diener and Bregman, 1998a). In order to execute this task successfully, a rat must reach through a partition that separates it from a food reward, and retrieve that reward. The testing apparatus is designed to allow only one paw through the partition at a time so that the rat cannot use two paws to scoop up the food. Therefore, the rat must be able to actually grasp the reward in order to perform the task successfully, a task that necessitates both fine and skilled motor control.

Advances in research

Although CNS axons attempt to regrow following SCI, regeneration and reinnervation in the spinal cord has not been successful (Schwab, 1996; Nicholls et al., 1999). In the PNS, which has the capacity to regenerate (Fawcett and Keynes, 1990; Raivich and Kreutzberg, 1993b), there is rapid neuronal degeneration and myelin clearance that is not found in the CNS. Since myelin contains molecules that are inhibitory to axonal growth, the delay in myelin clearance following CNS injury increases the concentration of these inhibitory molecules and decreases regrowth (Schwab et al., 1993; Thallmair et al., 1998). There is also a lack of regeneration-promoting substances, such as neurotrophic factors, in the CNS (Nakamura and Bregman, 2001; Widenfalk et al., 2001).

Experiments investigating different therapies that have modified the spinal cord milieu following injuries have resulted in more growth permissive environments that enhance regeneration.

Antibodies against growth inhibitors. Research has led to the characterization of proteins expressed by oligodendrocytes that inhibit the regrowth of axons after CNS injury (Caroni et al., 1988; Caroni and Schwab, 1988a, b; Schwab and Caroni, 1988). The antibody IN-1, raised against these proteins, neutralizes their inhibitory activity (Schwab, 1990a, b). Administration of IN-1 to the cerebrospinal fluid of adult rats after SCI resulted in a 5-18 mm elongation of CST axons caudal to the lesion, compared to only 1 mm elongation in the controls (Schnell and Schwab, 1993). Application of these antibodies also improved motor function (Bregman et al., 1995; Merkler et al., 2001). IN-1 used in combination with the neurotrophic factor, neurotrophin-3 (NT-3) after SCI promoted increased regeneration of the CST for up to 20 mm (Schnell et al., 1994).

Neurotrophic factors. While neurotrophic factors and their corresponding receptors have been found in the developing and adult spinal cord (Widenfalk et al., 2001), endogenous changes in these growth factors in response to SCI are not sufficient to result in regeneration. However, a number of studies have demonstrated that the exogenous application of growth factors to the lesioned spinal cord can promote regeneration. Axonal regrowth and functional improvements in grid walking resulted from the application of NT-3, produced by genetically modified fibroblasts (Grill et al., 1997) or supplied in a collagen matrix

(Houweling et al., 1998). Brain-derived neurotrophic factor (BDNF), both alone (Diener and Bregman, 1994) and in combination with embryonic spinal cord transplants (Bregman et al., 1998) rescued red nucleus neurons in newborn rats after spinal cord lesions. Both BDNF and NT-3 increased the growth of serotonergic, noradrenergic and CST axons in adult rats when combined with embryonic spinal cord transplants (Bregman et al., 1997a). When used in combination with Schwann cells grafts, BDNF and NT-3 enhanced the regeneration of propriospinal axons after SCI in adult rats (Xu et al., 1995b).

Transplants. The transplantation of various types of tissues and cells into the injured spinal cord has been investigated for a century (Schwab and Bartholdi, 1996). Research over the last 20 years has demonstrated that a number of different transplantable materials have the ability to enhance regrowth, both when used in combination with other therapies and alone.

Embryonic tissue and cells. As indicated above, pieces of fetal spinal cord tissue have been used successfully in combination with various neurotrophic factors to support regrowth. When utilized alone, fetal spinal cord transplants supported the regrowth of CST, raphespinal and rubrospinal axons (Diener and Bregman, 1998b) as well as promoted functional recovery after injuries to the neonatal spinal cord (Diener and Bregman, 1998a). The regenerative abilities of murine embryonic stem cells have also been investigated, and transplantation of these cells into lesioned adult rat spinal cords enhanced weight bearing and coordination of the hindlimbs (McDonald, 1999). While this area of research shows promise as a clinical therapy, there are a number of ethical issues

surrounding the use of human embryonic tissues that must be resolved prior to its use in treating human beings.

Peripheral tissue and cells. It is the ability of the PNS to regenerate that has inspired the use of components derived from the PNS to promote regeneration in the CNS. Peripheral nerve segments, used as “bridges”, allowed for axonal elongation into areas of the spinal cord caudal to the lesion (David and Aguayo, 1981) and improved function (Cheng et al., 1996). However, because central axons, like peripheral axons, attempt to regrow after lesioning, the problem with regeneration appears not to be with the “nerves”, but may be with the glial cells surrounding them. Therefore, research has been pursued investigating the ability of the peripheral glial cell that surrounds PNS axons, called a Schwann cell, to promote regeneration in the CNS. Initial studies demonstrated that mixed cultures of peripheral neurons and Schwann cells transplanted into the lesioned dorsal funiculus promoted regrowth and elongation of the CST in neonatal rats (Kuhlengel et al., 1990). Purified suspensions of Schwann cells injected into the dorsal funiculus after focal electrolytic lesioning in adult rats induced axonal sprouting of ascending and descending fibers (Li and Raisman, 1994). The transplantation of cultured Schwann cells at various time points after injury has been examined (Martin et al., 1996), and it was demonstrated that transplantation was most effective when performed immediately after lesioning, resulting in reduced gliosis, improved Schwann cell survival, and regeneration of axons from the dorsal root ganglion. After complete transection, spinal cord stumps inserted into guidance channels containing

Schwann cells showed axonal regrowth into the channels. However, axons did not regenerate into the lesioned spinal cord (Xu et al., 1997a). When the corticosteroid, methylprednisolone, was used in combination with Schwann cell-seeded guidance channels after spinal cord transection, supraspinal brainstem neurons extended axons into the channels, a result not observed when the channels were used alone (Chen et al., 1996). Grafts of human Schwann cells, seeded into guidance channels, were transplanted into transected rat spinal cords after the graft was removed from the channel. When this therapy was used in combination with methylprednisolone, it resulted in the regrowth of axons beyond the graft into the host spinal cord and modest functional improvement (Guest et al., 1997a). While grafts of Schwann cells encourage axonal regrowth and improve function, Schwann cells don't thrive well when transplanted into the CNS. The ideal candidate for transplantation may be a cell type that endogenously exists in the CNS, but is able to promote regeneration, as seen in the PNS.

Ensheathing cells. One glial cell type recently explored for its potential to promote regeneration in the damaged spinal cord is the ensheathing cell, derived from the olfactory epithelium. Ensheathing cells exclusively enfold olfactory axons (Doucette, 1991). It is believed that the ability of olfactory axons to reinnervate the adult mammalian olfactory bulb throughout an organism's lifetime is a result of the presence of this unique glial cell in the olfactory system (Doucette, 1995). Ensheathing cells, which are found in both the PNS and CNS (Doucette, 1991), are neither Schwann cells nor astrocytes, but instead a glial

cell that is unique to the olfactory system (Doucette, 1984). Some characteristics of ensheathing cells include their ability to: become more astrocyte-like or more Schwann cell-like as the need arises (Doucette, 1995); endogenously exist in both the PNS and the CNS (Doucette, 1990); co-exist with astrocytes (Franklin and Barnett, 1997), and remyelinate CNS axons both *in vitro* (Devon and Doucette, 1992) and *in vivo* (Franklin et al., 1996). All these properties made ensheathing cells a likely candidate to promote axonal regeneration after transplantation into the injured spinal cord.

Ensheathing cells were first transplanted into the spinal cord in 1994. A dorsal root transection was performed in adult rats and the stump was anastomosed to the spinal cord at the same point the root had entered before cutting. Ensheathing cell suspensions were injected at the point where the injured root was anastomosed. At three weeks post-injury, regenerating fibers traversed the root-spinal cord junction, crossed the zone of Lissauer, and grew into the gray matter of the dorsal horn (Ramon-Cueto and Nieto-Sampedro, 1994).

Cultured, unpurified ensheathing cells have been injected into adult rat spinal cords, unilaterally lesioned by focal electrolysis of the CST. In one study, CST axons grew through the transplant and formed a continuous bridge, resulting in DFR on the lesioned side (Li et al., 1997). In a subsequent study, regenerating CST axons were found amassed into parallel bundles that crossed the lesioned area and reentered the spinal cord (Li et al., 1998).

The ability of ensheathing cells, used in combination with the Schwann cell-seeded guidance channels described above, to promote regeneration after complete spinal cord transection was investigated. Numerous immunoreactively distinct populations of axons traversed the glia scar formed at both spinal cord-graft interfaces and ascending propriospinal axons were observed to regenerate at least 25 mm into the rostral spinal cord. Transplanted ensheathing cells, co-localized with regenerating axons, migrated from the injection sites, reaching 15 mm, and invaded the Schwann cell and connective tissue bridges as well as the adjacent white matter, gray matter, and glial scars (Ramon-Cueto et al., 1998).

When used without any other therapy, ensheathing cells transplanted into the completely transected spinal cords of adult rats resulted in the regeneration of CST, raphespinal, and coeruleospinal axons for at least 10 mm, and up to 30 mm, caudal to the lesion. This regeneration resulted in significant functional recovery of the hindlimbs, including locomotion, weight bearing, and response to light touch (Ramon-Cueto et al., 2000).

In light of these data, ensheathing cells are now recognized as a valuable tool in promoting spinal cord regeneration in animal models (Bartolomei and Greer, 2000; Franklin and Barnett, 2000; Raisman, 2001; Treloar et al., 2001) and the feasibility of transplanting ensheathing cells into human beings is beginning to be explored. Ensheathing cells from pigs, an ethically acceptable source of transplant material for human beings, have been genetically engineered to express a human complement inhibitory protein, in order to decrease, but not eliminate, rejection when transplanted into human beings

(Imaizumi et al., 2000). The pig ensheathing cells, transplanted into adult rats after dorsal column transection, promoted axonal regeneration, elongation, and remyelination, and restored impulse conduction across the lesion site. Ensheathing cells also migrated several millimeters along the rostral-caudal axis of the spinal cord (Imaizumi et al., 2000).

The demyelinated spinal cords of adult rats were injected with human ensheathing cells, obtained from adult patients undergoing olfactory nerve resection. Throughout the lesion site, extensive remyelination, characteristic of ensheathing cells, was observed. These results further demonstrate that ensheathing cells are a prime candidate for treating spinal cord injuries in human beings (Kato et al., 2000).

Corticosteroids. The natural adrenocortical hormones are steroid molecules produced and released by the adrenal cortex. Hormonal steroids are classified into two categories: those having important effects on intermediary metabolism (glucocorticoids) and those having principally salt-retaining activity (mineralocorticoids). In humans, the most important endogenous glucocorticoid is cortisol. Upon entering a cell, glucocorticoids bind to the cytoplasmic glucocorticoid receptor (GR), which is inhibited from migrating to the nucleus by a bound heat-shock protein (HSP 90). When a glucocorticoid binds to the GR-HSP 90 complex, HSP 90 is released, and the glucocorticoid-GR complex is then transported to the nucleus. In the nucleus, the glucocorticoid-GR complex acts as a glucocorticoid response element on various genes and regulatory proteins

(cell-type specific) to stimulate or inhibit their expression (Goldfien, 1995), resulting in numerous changes, some of which are described below.

Glucocorticoids have the capacity to dramatically reduce the manifestations of inflammation that results in five characteristic external symptoms: redness, swelling, heat, pain, and loss of function. After a single dose of a glucocorticoid, the concentration of lymphocytes, monocytes, eosinophils, and basophils in the circulation decreases, leading to a reduction in the number of cells at the site of inflammation. Glucocorticoids also inhibit the functions of leukocytes and tissue macrophages, reducing the ability of these cells to respond to antigens and mitogens. Glucocorticoids are also able to affect the inflammatory response by reducing the prostaglandin and leukotriene synthesis that results from activation of phospholipase A_2 . Glucocorticoids enhance the expression of the gene for lipocortin, which inhibits phospholipase A_2 and thus prevents the liberation of arachidonic acid and the synthesis of prostaglandins and leukotrienes. Glucocorticoids also inhibit gene expression of cyclooxygenase-2, thus reducing the amount of enzyme available to produce prostaglandins. In addition, glucocorticoids cause vasoconstriction when applied directly to blood vessels, and decrease capillary permeability by inhibiting the activity of kinins and bacterial endotoxins and by reducing the amount of histamine released by basophils (Goldfien, 1995).

Methylprednisolone is a synthetic glucocorticoid steroid that was initially developed for its enhanced anti-inflammatory activity and lessened mineralocorticoid activity compared to cortisol (Hall, 1992). Relative to cortisol,

methylprednisolone is about four times as potent as an anti-inflammatory agent, with only 0.8 times the mineralocorticoid activity (Hall, 1992). The plasma half-life of methylprednisolone is approximately 2.5 hours, however, the biological half-life of methylprednisolone is 12 to 36 hours (Hall, 1992) because its mechanism of action results in changes in protein synthesis that develop slowly over days and persist much longer than the half-life of methylprednisolone in the blood. Methylprednisolone is an extremely lipophilic molecule, which is a drawback to its intravenous use, however, this has been overcome by the production of a methylprednisolone-hemisuccinated ester (Solu-Medrol). When Solu-Medrol is injected into the body, the ester is liberated from the pro-drug, primarily by liver esterases, releasing the free steroid, which is believed to cross the blood brain barrier, and is responsible for the neuroprotective effects exhibited by this drug (Hall, 1992).

While glucocorticoids have been used in the clinical treatment of spinal cord trauma since the 1960's, the rationale for their use primarily centered on the expectation that they would reduce post-traumatic spinal cord edema. It was the extensive research in the 1970's and 1980's demonstrating the effects of methylprednisolone on secondary injury after acute experimental SCI (Hall et al., 1978; Green et al., 1980; Braughler and Hall, 1982a, b; McGinley et al., 1982; Braughler and Hall, 1983; Bracken et al., 1984; Braughler and Hall, 1984; Hall et al., 1984), that led to the first (Bracken et al., 1984), second (Bracken et al., 1990), and third (Bracken et al., 1997) National Acute Spinal Cord Injury Study (NASCIS).

In the first NASCIS (Bracken et al., 1984), methylprednisolone was administered to 330 patients with acute SCI. The patients received a bolus injection of either 1,000 mg or 100 mg Solu-Medrol, followed by either 250 mg or 25 mg, respectively, every six hours for ten days. No difference was observed between the two groups in neurological recovery of motor function or pinprick and light touch sensation at either six weeks or six months after injury. However, since no control group was used in this study, no comparison could be made between treated and untreated patients to determine if methylprednisolone had any effect on these behavioral parameters. In the second NASCIS (Bracken et al., 1990), a placebo-controlled group was included. Patients treated with methylprednisolone (162 patients) within eight hours of injury had significant improvements in motor function and sensation to pinprick and touch when compared to placebo controls (171 patients). In the third NASCIS (Bracken et al., 1997) the efficacy of methylprednisolone administered for 24 hours (166 patients) was compared with the efficacy of methylprednisolone administered for 48 hours (167 patients). The data from this study indicated that patients initiating methylprednisolone treatment within 3 hours of injury should be maintained on the treatment regimen for 24 hours post-injury, while patients initiating treatment 3 to 8 hours after injury should have their maintenance dose extended to 48 hours.

Any treatment that has the potential for use in human beings might be examined in combination with methylprednisolone, as it is the only therapy currently used in human beings after SCI. No one has previously examined the

combined effects of methylprednisolone and ensheathing cells in any species. The aim of this research was to explore the potential regenerative properties of the combined treatment of ensheathing cells and methylprednisolone after acute injury in the adult rat spinal cord. The hypothesis was that a combination of interventions consisting of the transplantation of ensheathing cells followed by administration of methylprednisolone promotes regeneration and functional reinnervation of CST axons after acute cervical spinal cord injury in adult rats. The experimental models that were used to test this hypothesis included: an *in vitro* model to purify and characterize ensheathing cells; a behavioral testing model modified to examine CST function after bilateral lesioning; and an *in vivo* model of spinal cord injury in the adult rat. These model systems were utilized to characterize a purified population of ensheathing cells, which was used in combination with methylprednisolone to repair the lesioned CST in rats. The effectiveness of these therapies, both alone and in combination, were examined functionally and morphologically. The long term objective of research studies such as this one is to eventually find a cure for the debilitating deficits that result from SCI.

CHAPTER 2

A New Method Of Purification For Establishing Primary Cultures Of Ensheathing Cells From The Adult Olfactory Bulb

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Abstract

Ensheathing cells exclusively enfold olfactory axons. It is believed that the ability of olfactory axons to reinnervate the adult mammalian olfactory bulb throughout an organism's lifetime is a result of the presence of this unique glial cell in the olfactory system. This theory has been substantiated by research demonstrating the ability of transplanted ensheathing cells to promote axonal regrowth in areas of the central nervous system which are normally non-permissive. A simple method for purifying ensheathing cells resulting in a large yield of cells is therefore invaluable for transplantation studies. We have developed such a method based on the differing rates of attachment of the various harvested cell types. The greatest percentage of cells (70.4%) that attached during the first step of the separation were determined to be fibroblasts. The remainder of the cells were classified as astrocytes (20.8%) and ensheathing cells (6.8%). The percentage of attached astrocytes (67.6%) was greatly increased during the second purification step while the percentage of fibroblasts decreased greatly (27.9%) and the percentage of ensheathing cells (5.3%) slightly decreased. In the final cultures, 93.2 % of the attached cells were ensheathing cells, while astrocytes (5.9%) and fibroblasts (1.4%) were only minor components. This simple, inexpensive method of purifying ensheathing cells will facilitate their use in central nervous system regeneration research.

Introduction

Primary olfactory neurons, which have a life span of approximately 4 weeks (Graziadei and Monti-Graziadei, 1978), are unique in the adult mammalian nervous system, as they are in a state of continuous turnover. When these neurons die, they are replaced by new neurons originating from neuroepithelial precursors located in the olfactory epithelium (Graziadei and Monti-Graziadei, 1978). The growing primary olfactory axons successfully reinnervate the olfactory bulb and form functional synapses with mitral cells within the bulb (Kosaka et al., 1998).

In general, axons in the peripheral nervous system (PNS) are enfolded and myelinated by Schwann cells (Fraher, 1999). As axons exit the PNS and enter the central nervous system (CNS), they cross through the CNS-PNS transition zone, and are then supported by astrocytes and myelinated by oligodendrocytes (Fraher, 1999). This transition is not found in the olfactory system (Doucette, 1991). Instead, olfactory axon fascicles are ensheathed throughout the PNS (Doucette, 1991) and into the CNS (Doucette, 1993b) by a macroglial cell termed ensheathing cells (EC, (Doucette, 1993b)).

Methods have been developed to harvest and purify EC. EC have been obtained from embryonic tissue (Doucette, 1993a), when the nerve fiber layer is only loosely attached to the marginal zone of the primordial olfactory bulb (Doucette, 1993b) and from neonatal tissue using fluorescence-activated cell sorting (Barnett et al., 1993). Two procedures describing the purification of EC from adult rats have been reported. One study utilized immuno-panning

(Ramon-Cueto and Nieto-Sampedro, 1994) while the other study purified EC using magnetic beads (Gudino-Cabrera and Nieto-Sampedro, 1996). These methods result in varying degrees of ensheathing cell purity and yield.

Currently a number of laboratories are exploring the potential for transplanted EC to promote axonal regeneration in the adult mammalian CNS after injury. Following ablation of the fimbria-fornex, implanted EC were found to survive and support the regrowth of cholinergic axons (Smale et al., 1996). Transplanted EC promoted regrowth of corticospinal axons and improved function in the forelimbs after electrolytic lesioning of the corticospinal tract (Li et al., 1997). After complete spinal cord transection, sensory axons traveled 1.5 cm past the lesion after transplantation of EC and Schwann cells (Ramon-Cueto et al., 1998) and sensorimotor reflexes and locomotor functions were recovered after transplantation of EC (Ramon-Cueto et al., 2000). Recently, transplanted transgenic porcine ensheathing cells were found to induce axonal regeneration and remyelination as well as restore impulse conduction in lesioned adult rat spinal cords (Imaizumi et al., 2000).

To support and advance this exciting ensheathing cell research, it is necessary to have a method by which EC can be easily and inexpensively purified. We have developed a method to isolate EC from adult rats that does not bind any antibodies or require any expensive equipment, and yields a high quantity of cells from adult animals. Some of these results were reported in abstract form at the 1999 Society for Neuroscience Annual Meeting (Nash et al., 1999).

Materials and Methods

Cell Cultures:

Ninety adult Sprague Dawley rats (200-250 g, Taconic Farms, Germantown, NY) were used in this study. For the establishment of primary cultures, rats were anesthetized with 10% chloral hydrate (10 ml/kg, Sigma, St. Louis, MO) and decapitated. A skin incision was made in the scalp extending from the tip of the nose to the occipital bone of the skull and the nasal and frontal bones were removed. The olfactory nerve rootlets and olfactory bulbs were detached and placed in 10 ml of chilled calcium and magnesium free Hanks Balanced Salt Solution (HBSS, Biofluids, Rockville, MD). The HBSS was kept on ice during harvesting.

The tissue was prepared for seeding into chamber/slides, based on the technique described by Ramón-Cueto and Nieto-Sampedro (Ramon-Cueto and Nieto-Sampedro, 1992). After harvest, the tissue was divested of all meninges and blood vessels and placed in 10 ml of chilled HBSS. The outer two layers of the olfactory bulb, the olfactory nerve and glomerular layers, were dissected and retained. These two layers and the olfactory rootlets from all animals were pooled in 10 ml of chilled HBSS. The tissues were minced with a razor blade, triturated using a 20 gauge needle and trypsinized (0.1% w/v Type IX trypsin (Sigma), 15 min each, 37°C, 5% CO₂). The trypsinization was quenched using a complete media consisting of Dulbecco's Minimum Essential Media (DMEM, Biofluids) and Ham's F-12 (F-12, Biofluids), at a 1:1 mixture with 10% fetal bovine serum (Biofluids), 1% glutamine (Biofluids), 2% penicillin-streptomycin

(Biofluids) and 1% gentamicin (Biofluids). The cells were centrifuged twice (1000 r.p.m., 10 min). To ascertain the number of viable cells, the pellet was suspended in 1 ml complete media and one drop of trypan blue mixed with one drop of the cell suspension was placed on the surface of a hemocytometer. Viable (unstained) cells were counted and three pairs of olfactory bulbs yielded approximately 5.1×10^6 cells. Complete media was added to yield a suspension containing 8.5×10^5 cells/ml.

Purification of Cultures:

The technique used for purification of EC is diagrammed in Figure 1. Both glass and plastic slides were examined (Lab-Tek, Naperville, IL) and there were no noticeable differences. The cell suspension was seeded into three uncoated chamber/slides (Step 1 slides) and incubated (18 hr, 37°C and 5% CO₂). Most of the fibroblasts attach to the Step 1 slides during this first incubation period since fibroblasts will settle within 1 hr of seeding (Polinger, 1970). The supernatants from the Step 1 slides were poured into a second set of three uncoated slides (Step 2 slides). The Step 2 slides were incubated (36 hr, 37°C and 5% CO₂) to allow for the attachment of astrocytes. When preparing primary cultures of olfactory bulbs, we had observed that EC did not attach to uncoated slides for 96 - 120 hr. Therefore, at 36 hr of incubation, the majority of the EC remained in the supernatant. This supernatant was used to seed a set of three poly-L-lysine (Sigma) coated slides (Step 3 slides). After seeding, the EC attached to the Step 3 slides within 48 hr. As has been reported previously, neurons do not survive this culture environment (McCarthy and de Vellis, 1980). All slides were

maintained in an incubator (37°C, 5% CO₂) for eight days after purification, and the media was changed every two days. Three to nine Step 1 slides were prepared for each experiment, and the experiments were replicated numerous times.

Immunofluorescence Labeling:

Thy 1.1 antibodies are routinely used to label fibroblasts in culture (Stern, 1973). The p75 antibody labels EC (Ramon-Cueto and Nieto-Sampedro, 1992), while the antibody against glial fibrillary acidic protein (GFAP) labels both EC (Ramon-Cueto and Nieto-Sampedro, 1992) and astrocytes (Bignami and Dahl, 1973). Three primary antibodies were used to characterize these cultures. Mouse anti-rat p75 (Boehringer Mannheim, Indianapolis, IN) and mouse anti-Thy 1.1 (Serotec, Oxford, U.K.) are monoclonal IgGs used at dilutions of 1:200 and 1:1200, respectively. These primary antibodies were diluted in 1% normal goat serum (DAKO, Carpinteria, CA) and 0.1% bovine serum albumin (Sigma) in a 1:1 mixture of DMEM and F-12 (DMEM/F-12 solution). The third primary antibody, a polyclonal rabbit anti-cow GFAP IgG (DAKO) was used at a dilution of 1:500 in phosphate buffered saline (PBS, Sigma) with the addition of 1% normal goat serum and 0.1% bovine serum albumin (PBS solution). Two secondary antibodies were used: Cy3-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:200 in the DMEM/F-12 solution and fluorescein-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) at a dilution of 1:50 in the PBS solution.

Cultured cells from each purification step were immunolabeled with one of these primary antibody combinations: anti-p75 and anti-GFAP or anti-Thy 1.1 and anti-GFAP. Double labeling with anti-p75 and anti-Thy 1.1 was performed even though the only primary antibodies available to label these molecules in rat tissue are mouse monoclonals. Realizing the problems associated with double labeling using these monoclonals, a sequential labeling procedure was utilized, however the results were not interpretable and will not be further described.

All cultures were washed (DMEM/F-12, 4X, 5 min each) and treated to block non-specific binding (DMEM/F-12, 1 hr, 37°C, 5% CO₂). One set of cultures from each purification step received anti-p75, while a different set of cultures from each purification step received anti-Thy1.1. Negative controls did not receive a primary antibody. All cultures were incubated (1 hr, 24°C) and washed (DMEM/F-12, 4X, 5 min each).

The Cy3-conjugated goat anti-mouse secondary antibody was applied to the cultures and incubated (37°C, 5% CO₂, 1 hr). After incubation, cultures were washed (PBS, 1X, 2 min, 24°C) fixed (4% paraformaldehyde, 30 min, 24°C) and then washed again (PBS, 4X, 5 min each, 24°C). Cultures were washed (PBS solution plus 0.1% Triton-X (Sigma), 4X, 5 min each, 24°C), incubated overnight (4°C) in anti-GFAP (except negative controls) and washed again (PBS solution, 4X, 5 min each, 24°C). All cultures were incubated (1hr, 24°C) in fluorescein-conjugated goat anti-rabbit IgG and washed (PBS, 4X, 5 min each, 24°C).

Results

The purity of the cell cultures and number of EC obtained was determined by counting the immunofluorescently labeled cell types attached to the slides. The 1 mm² sample area was randomly placed over the slide (total area 900 mm²) and all cell types within that area were counted. Thirty sample areas per slide were used to calculate the average number of each cell type, expressed as percentages.

The following labeling criteria were used to identify cell types from each purification step and the final cultures: cells labeled with anti-Thy 1.1 alone were designated as fibroblasts; cells labeled with only anti-GFAP were designated as astrocytes; and EC were identified by co-labeling with anti-p75 and anti-GFAP (Fig. 2).

Thy 1.1 is a fibroblastic cell membrane glycoprotein. Antibodies against Thy 1.1 bound to fibroblasts, resulting in a punctate appearance on the cell surface which was most dense in cell processes and membrane margins (Fig 2A). The fibroblast morphology varied from round to endothelial-like to spindle-shaped.

The antibody to GFAP labels both EC (Ramon-Cueto and Nieto-Sampedro, 1992) and astrocytes (Bignami and Dahl, 1973), while p75 is the definitive marker of EC in olfactory cultures (Ramon-Cueto and Nieto-Sampedro, 1992). The intermediate filaments of astrocytes are comprised of GFAP and can be seen to project from a peri-nuclear ring, through the cytoplasm to the cell membranes of astrocytes and define their shape (Fig. 2B). As with astrocytes,

the intermediate filaments of EC are composed of GFAP, and are therefore GFAP immunoreactive (Fig. 2C). However, unlike the intermediate filaments of astrocytes which are large, closely packed bundles (Fig. 2B), the individual, GFAP-positive intermediate filaments of EC are loosely scattered throughout the cytoplasm (Fig. 2C). Co-labeling of GFAP and p75 antibodies is seen in EC (Fig. 2C). As p75 is a receptor found on the external surface of the plasma membranes of EC, labeling with antibodies against p75 results in punctate labeling on the surface of these cells (Fig. 2C).

The three immunofluorescently labeled and identified cell types were counted using the criteria described above. The average numbers of attached fibroblasts, astrocytes, and EC were determined and expressed as percentages. This analysis was performed for each of the purification steps (Step 1 and Step 2) and the final cultures (Step 3). The relative percentages of the three cell types varied dramatically from step to step and reflected the dominant cell type found at each step (Fig. 3).

The greatest percentage of cells (70.4%) that attached during Step 1 were labeled with Thy 1.1 alone, and were therefore determined to be fibroblasts. The remainder of the cells were classified as astrocytes (20.8%) and EC (6.8%) (Fig. 3). The percentage of attached astrocytes (67.6%) was greatly increased during the second purification step (Step 2). The percentage of attached fibroblasts decreased to 27.9% while the percentage of EC (5.3%) decreased slightly (Fig. 3). In contrast, 93.2 % of the attached cells in the final cultures were EC. Astrocytes (5.9%) and fibroblasts (1.4%) were only minor components of these

final cultures (Fig. 3). These results demonstrate that a large population, approximately 1.02×10^6 EC per rat, with a purity of greater than 90% was obtained by this separation procedure.

Analysis of the immunocytochemically labeled cells revealed an unexpected finding. Thy 1.1 immunoreactivity was found to be co-localized with GFAP immunoreactivity (Fig. 2D). Although a low percentage of astrocytes (10%) are known to express Thy 1.1 after 7 days in culture (Pruss, 1979), EC have not been previously reported to express this molecule. When the number of cells expressing Thy 1.1 and GFAP was determined for each of the purification steps, the percentage of these cells were similar, but slightly higher than the percentage of EC expressing both p75 and GFAP. In the Step 1 cultures, the percentage of cells co-expressing Thy 1.1 and GFAP was 7.9%. In Step 2 cultures, 8.2% of the cells co-expressed Thy 1.1 and GFAP, while in the final cultures, the percentage of these cells increased to 93.6%. Based on this numerical evidence, EC are hypothesized to express p75, Thy 1.1 and GFAP (Nash et al., 1999). Like p75, Thy 1.1 expression on EC is restricted to the surface of their plasma membranes. As a result, EC labeled with anti-p75 and anti-GFAP (Fig. 2C) or anti-Thy 1.1 and anti-GFAP (Fig. 2D) have very similar patterns of punctate labeling.

Discussion

Purification:

Two procedures describing the purification of EC from adult rats have previously been reported. One study, which utilized immuno-panning, incubated the dissociated tissue in plates pre-coated with anti-p75 antibodies (Ramon-Cueto and Nieto-Sampedro, 1994). As EC are the only cells in the harvested tissue that express p75, all other cell types are washed off, and a purified (90%) population of EC is obtained. When we attempted this method, however, the yield of EC after the purification was so low that cells didn't attach after seeding into culture flasks. In contrast, our method results in the yield of a large number of EC (1.02×10^6 /rat). In addition, unlike immuno-panning, our method does not require the use of antibodies which could potentially interfere with subsequent immunolabeling and the normal functioning of the cells.

The other previously reported study also harvested tissue from adult rats and purified EC using magnetic beads (Gudino-Cabrera and Nieto-Sampedro, 1996). The harvested, dissociated cells were suspended with magnetic beads coated with anti p75-antibodies. Non-attached cells were washed away resulting in purified (97%) populations of EC. In addition, this procedure resulted in the retention of 1000-fold more EC than the immuno-panning method. However, even after multiple washings, medium changes during culturing, and transplantation *in vivo*, immuno-magnetic beads were still observed attached to EC one month after the purification procedure (Gudino-Cabrera and Nieto-

Sampedro, 1996). Clearly, the fact that our method does not result in the binding of any additional molecules or particles to the EC makes it advantageous.

The underlying principle for this method of separation is that the cell types harvested from the outer two layers of the adult olfactory bulb have different rates of attachment to various substrates. Fibroblasts settle within 1 hr of seeding onto uncoated slides (Polinger, 1970), leaving the majority of astrocytes and EC in the supernatant. Transferring the supernatant to another uncoated slide and incubating for 24 hr allows attachment of the astrocytes. EC, which remain in suspension, then settle onto the poly-L-lysine coated slides within 24-48 hr, and are 93% pure (Nash et al., 1998).

This method to isolate and characterize EC from adult rats was developed to procure purified EC for transplantation into injured rat spinal cords. EC appear to retain the essential molecular properties necessary to promote axonal elongation in the CNS after harvest and transplantation. A number of regeneration studies have transplanted both purified and non-purified EC. Transplanted EC were found to support regenerating dorsal root axons into a rhizotomized spinal cord (Ramon-Cueto and Nieto-Sampedro, 1994) and facilitate axonal elongation in adult rat brain after fimbria-fornix transection (Smale et al., 1996). Non-purified EC promote axonal elongation into and beyond corticospinal tract lesions (Li et al., 1997, 1998) corresponding to the migrational path of the transplanted olfactory cells. Partial recovery of corticospinal tract function was also reported (Li et al., 1997). ECs have promoted axonal elongation for 1.5 cm in completely transected spinal cords

(Ramon-Cueto et al., 1998). Transgenic EC from pigs were transplanted into the spinal cords of adult rats after lesioning the dorsal columns and found to promote axonal regeneration, remyelination, and restoration of impulse conduction across lesion site (Imaizumi et al., 2000).

Although it is unlikely that a single therapeutic intervention can correct all of the problems associated with CNS injury, what is necessary is a cell type that can assume many diversified roles during the attempted regeneration by the CNS. EC are ideally suited to fulfill this role. Therefore the development of an easy, inexpensive technique resulting in a high yield of purified EC is invaluable.

EC and Thy 1.1:

Thy 1.1 has been a protein in search of a function since it was identified in 1964 (Reif and Allen, 1964). This 25 kD glycoprotein (Kroczeck et al., 1986) has been found on the surfaces of thymocytes (Reif and Allen, 1964), neurons (Mirsky and Thompson, 1975), epidermal cells (Scheid et al., 1972) and fibroblasts (Stern, 1973) *in vivo* and *in vitro*.

Although astrocytes do not express Thy 1.1 *in vivo*, 10% of astrocytes exhibit Thy 1.1 by 7 days in primary brain cultures (Pruss, 1979; Raff et al., 1979). It is therefore probable that a small percentage of cells in each of the culture steps labeled with antibodies against both GFAP and Thy 1.1 are astrocytes. This is supported by the fact that the percentage of cells doubled labeled with Thy 1.1 and GFAP antibodies (Step 1 - 7.9%, Step 2 – 8.2%; and Step 3 – 93.6%) was similar, but always slightly higher than the percentage of cells labeled with p75 and GFAP antibodies (Step 1 – 6.8%, Step 2 – 5.3%; and

Step 3 – 93.2%). When the cells in Step 2 were examined, the percentage demonstrating Thy 1.1 and GFAP immunoreactivity versus p75 and GFAP immunoreactivity was a bit higher when compared with those in Steps 1 and 3. This increase was due to the fact that the majority of astrocytes attach during the second purification step and 10% of astrocytes exhibit Thy 1.1 labeling by 7 days in culture.

The similarity between the percentages of cells double labeled with anti-p75 and anti-GFAP and the cells labeled with anti-Thy 1.1 and anti-GFAP strongly suggests that EC are expressing Thy 1.1. If the labeling were a result of 10% of the astrocytes expressing Thy 1.1 (single labeling for GFAP was 22.5% for Step 1, 67.6% for Step 2, and 5.5% for Step 3), the resulting percentages of cells would be different than those actually observed.

Summary:

EC, which are unique to the olfactory system, are believed to be responsible for the ability of olfactory axons to innervate this area of the adult mammalian CNS over a lifetime. This hypothesis has been substantiated by research demonstrating the ability of transplanted EC to promote axonal regrowth in areas of the CNS which are normally non-permissive. Therefore, a simple method of purifying EC resulting in a large yield of cells for transplantation is invaluable. We have developed a method to isolate EC from adult rats based on the differential rates of attachment of fibroblasts, astrocytes and EC to a plastic or glass surface. Our technique results in a population of EC that are >90% pure. It does not require the use of any specialized cell sorting equipment

or the binding of antibodies which could potentially interfere with subsequent immunolabeling. This method is also economical, easy to perform, and it yields a large quantity of purified EC.

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Figure 1. Schematic of Purification Process

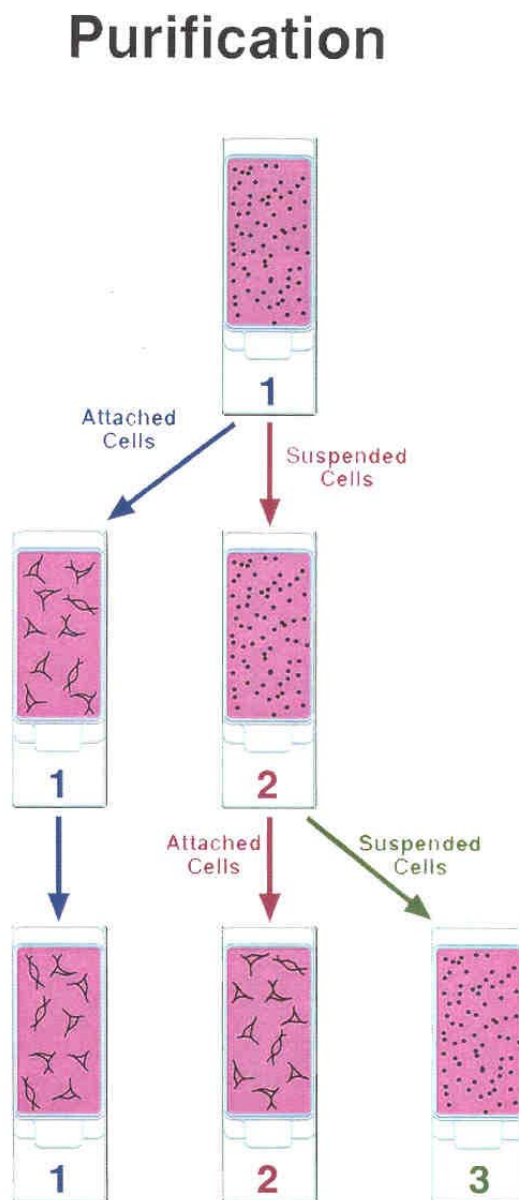


Figure 1: Scheme for purification of ensheathing cells. The initial cell suspension is placed in the uncoated Step 1 slides. Eighteen hours later, the cells which remain in suspension are transferred to the uncoated Step 2 slides. After incubation for 36 hours, the cells which remain in suspension are transferred to the poly L-lysine coated Step 3 slides.

Figure 2. Photomicrographs of Cultured, Immunolabeled Cell Types

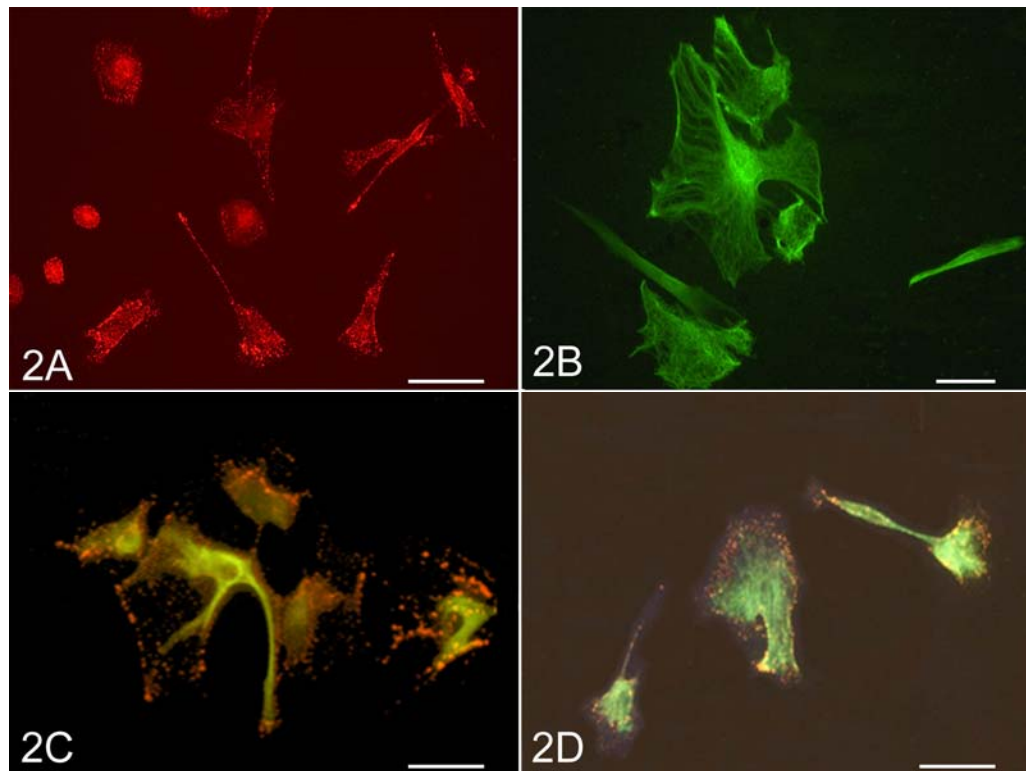


Figure 2: Photomicrographs of immunolabeled cell types cultured from layers 1 and 2 of the adult rat olfactory bulb. On Step 1 slides, the majority of cells show punctate Thy 1.1 immunoreactivity and are identified fibroblasts (2A). The preponderance of cells that attached to Step 2 slides are astrocytes (2B). These cells are identified by dense GFAP immunoreactivity in the long, tightly bundled intermediate filaments. The majority of cells that attached to the Step 3 slides are ensheathing cells co-labeled with either p75 and GFAP (2C) or Thy 1.1 and GFAP (2D). Thy 1.1 and p75 antibodies are secondarily linked to Cy3 (red) and GFAP antibodies are secondarily linked to fluorescein (green). p75 (2C) and Thy 1.1 (2D) are localized to the surface of the ensheathing cell membranes, unlike GFAP (2C and 2D), which is found in the loosely scatter intermediate filaments in the cytoplasm. Scale bars = 20 μ m.

Figure 3. Percentages of Immunolabeled Cell Types

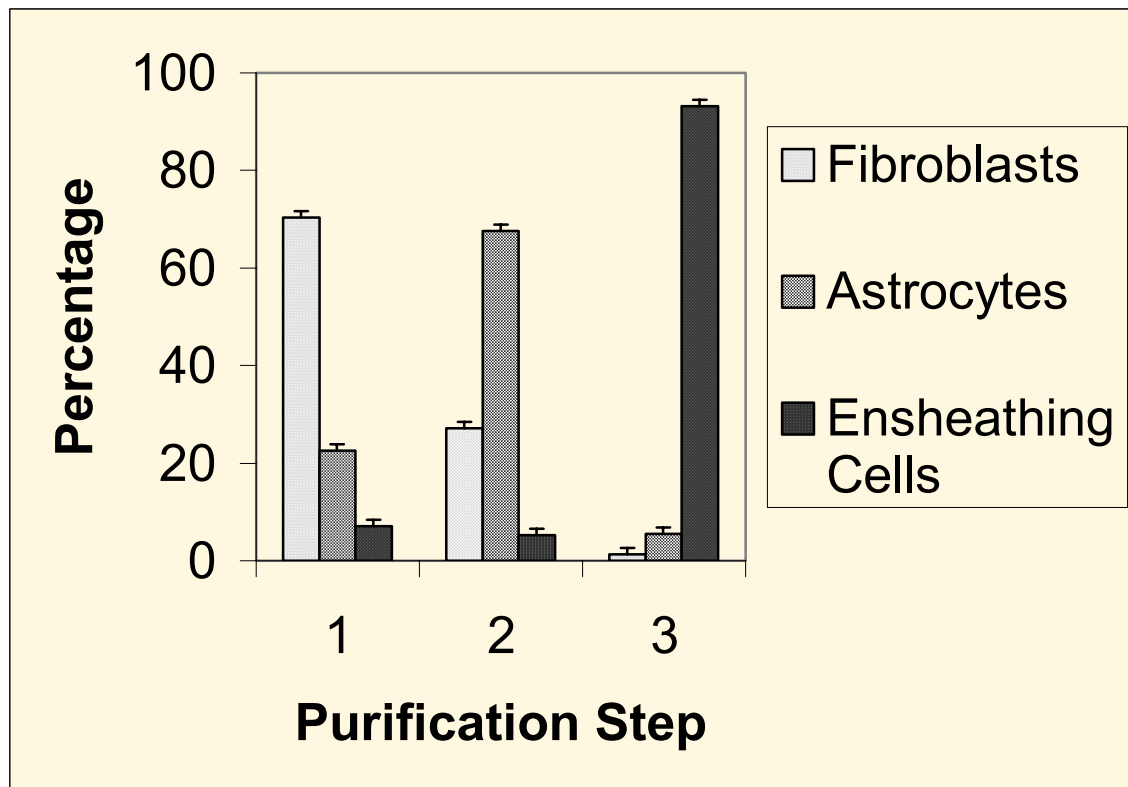


Figure 3: Quantity of each cell type (expressed as a percentage) attached during each purification step. In Step 1, 70.4 % of the attached cells were fibroblasts, 20.8% were astrocytes, and 6.8% were ensheathing cells. Of the cells that attached during step 2, 27.9% were fibroblasts, 67.6% were astrocytes, and 5.3% were ECs. In the final cultures (Step 3), only 1.4% of the attached cells were fibroblasts and 5.3% were astrocytes. The remaining 93.2% of attached cells were identified as ECs. Bars represent percentages of identified cell types \pm standard error of the mean.

CHAPTER 3

Directed Forepaw Reaching: An Improved Apparatus That Prevents Extinguished Behavior

submitted to Behavioral Brain Research

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Abstract

Damage to the corticospinal tract (CST) in humans can result in the loss of fine and skilled motor movements used in daily activities. Recently, there has been an increase in animal studies examining a multitude of treatment strategies to restore function after spinal cord axons, including those in the CST, have been injured. Successful evaluation of the effectiveness of these treatments requires functional assessment measures that provide quantitative data. Directed forepaw reaching (DFR) is a task often utilized to assess injury and recovery of the CST. Using the apparatus described by Castro (Castro, 1972b), we examined the ability of Sham Control and Lesion rats to perform the DFR task. Pre-surgically, rats in the Sham Control and Lesion groups performed the DFR task with a success level greater than 90% (96.2% and 95.3%, respectively). Post-surgically, the Sham Control rats still performed the DFR task at this high level (93.4%), while the Lesion rats were unable to perform the task (0%). We hypothesized that the abnormally low scores for Lesion rats were due to the extinguishing of the DFR behavior. To test our hypothesis, we modified the DRF apparatus in a way that prevented extinguishing of the behavior. The experiment was repeated on a new set of rats using the modified apparatus. As with the first group of rats, the Lesion and Sham Control rats performed the DFR task pre-surgically with a success level of greater than 90% (94.4% and 95.6%, respectively). The Sham Control animals tested in the modified apparatus had post-surgical score of 94.4%. However, with the modified apparatus, post-surgical scores for the Lesion rats increased to a success level of 39.9%,

demonstrating that the low scores initially obtained were the result of extinguished behavior and not an inability to perform the DRF task. These results demonstrate that by designing a modifiable functional apparatus to prevent the extinguishing of a learned behavior, researchers are assured that the behavioral data they obtain are not a reflection of flaws in the testing apparatus but are an accurate reflection of functional ability.

Introduction

It is estimated that 10,000 new cases per year of spinal cord injury occur in the United States alone (Center, 2001). Nationwide, there are over 200,000 people living with spinal cord injuries (Center, 2001). The functional loss of these individuals and the impact of the injury on their lives is an important clinical concern. An injury to the corticospinal tract (CST) results in the loss of fine and skilled motor movements. Even though CST axons attempt to regrow following injury, regeneration and reinnervation of spinal cord neurons is not successful, and the loss of function is presently irreversible.

Recently, there has been an increase in animal studies examining a multitude of treatment strategies for spinal cord injuries. Approaches to treating spinal cord injuries include pharmacological interventions (Hall et al., 1988; Bracken, 1991; Xu et al., 1998), application of neurotrophic factors (Fernandez et al., 1993; Schnell et al., 1994; Ye and Houle, 1997), blocking inhibitor proteins (Schnell and Schwab, 1990; von Meyenburg et al., 1998; Brosamle et al., 2000), and a variety of transplantation strategies (Joosten et al., 1995a; Guest et al., 1997a; Diener and Bregman, 1998a; Ramon-Cueto et al., 2000). Many of these treatments have been examined in combination with varying degrees of success (Schnell and Schwab, 1993; Chen et al., 1996; Guest et al., 1997b; Xu et al., 1997b; Bregman et al., 1998; Houweling et al., 1998; Ramon-Cueto et al., 1998). However, it is vital that the efficacy of these treatment strategies to promote recovery of function after spinal cord injury be assessed (Schallert et al., 2000). Behavioral testing procedures that permit the quantitative examination of

functional ability in animals with a normal or impaired function are crucial are crucial in the evaluation of these interventions.

Damage to the CST compromises fine and skilled movements while gross movements are unimpaired. These subtle deficits produced after injury to the CST requires appropriate testing methods to assess functional loss and recovery (Castro, 1972a) in the rat. Reaching, which involves the proximal musculature of the forelimbs is spared. Grasping, however, which involves distal portions of the forelimbs and digits, is impaired. A testing procedure that examines CST function in normal and injured rats and produces qualitative data is essential. The directed forepaw reaching (DFR) task, described by Castro in 1972 (Castro, 1972b), is such a method. In order to execute this task successfully, a rat must reach through a partition that separates it from a food reward, and retrieve that reward. The testing apparatus is designed to allow only one paw through the partition at a time so that the rat cannot use two paws to scoop up the food. Therefore, the rat must be able to actually grasp the reward in order to perform the task successfully, a task that necessitates both fine and skilled motor control.

DFR has been used in the examination of a variety of research areas that affect skilled forelimb movements (Price and Fowler, 1981; Whishaw and Tomie, 1989; Whishaw et al., 1993; Prusky and Whishaw, 1996; Whishaw and Coles, 1996; Pettersson et al., 1997; Z'Graggen et al., 1998; Schallert et al., 2000). Among its numerous uses, DFR has been utilized to examine changes in function after cervical spinal cord injury to the CST (Bregman et al., 1997b; Li et al., 1997; Pettersson et al., 1997; Diener and Bregman, 1998a, b; Muir and

Whishaw, 1999). In this study, the DFR apparatus described by Castro (Castro, 1972b), was utilized in these spinal cord injury experiments to examine functional changes after transection of the CST at cervical level 3.

Materials and Methods

Subjects:

Forty adult Sprague Dawley rats (200-250 g, Taconic Farms, Germantown, NY) were used in this study. Rats were randomly assigned to either the Sham Control or Lesioned group (10 rats/group), and the experiment was performed twice, once with an apparatus built to the specifications of Castro (Castro, 1972b), and once with a modified version of this apparatus. Animals were tested pre-surgically and post-surgically so that each animal served as its own control.

Apparatus:

Motor performance was measured in the first experiment in a box built as described by Castro (Castro, 1972b) and in the second experiment using a modified version of the apparatus.

Original Apparatus

The original box had two compartments separated by a Plexiglas divider. The two compartments included a main compartment for the rat and a minor compartment for the food reward (Fig.1A, 1B). The main compartment was 30 ½ inches wide by 6 ¼ inches deep by 5 inches high. The minor compartment was 30 ½ inches wide by 3 ½ inches deep by 3 inches high and was subdivided into 14 adjacent slots of equal size (2 inches wide, 1¼ inches deep, and 3 inches high). The Plexiglas divider separating the rat compartment from the food compartment was 30 ¾ inches wide by ¼ inch deep by 5 inches high, and had

14 semicircles drilled into the bottom, each of which was a $\frac{1}{2}$ inch in diameter. An important feature of this testing apparatus was an opening in the floor ($\frac{3}{4}$ inch) between the Plexiglas divider and the shelf on which the food reward was placed (Fig. 1A, 1C).

A single 190 mg food pellet (Bio-Serv, Frenchtown, NJ) was placed into each slot. Each rat was required to extend a forelimb through an opening in the Plexiglas divider to grasp a food pellet. The rats could use either forelimb to retrieve the food pellet, but the openings in the Plexiglas divider were only large enough to admit one limb at a time. The design of the apparatus required a rat to grasp and lift the food pellet, thus requiring fine and skilled motor control. If a rat attempted to scoop or rake a pellet out of the slot without grasping and lifting it, the pellet would fall irretrievably through the opening.

Modified Apparatus

The modification made to the original testing apparatus was that the shelf on which the food pellets were placed could be moved to close the opening in the floor (Fig. 1B, 1D). This feature is critical to any testing apparatus used with lesioned animals that are unable to successfully perform the retrieval task. If animals were unable to perform the task after lesioning due to impaired fine motor skills, then they would stop trying to perform the task and the behavior would extinguish. If the behavior extinguishes, the rats cannot be tested to determine the extent of functional recovery post-injury. By closing the opening in the floor for post-surgical animals who cannot perform the task for the duration of the recovery period until function was restored, the reaching behavior was

maintained, preventing extinguishing of the learned task and ensuring accuracy of testing.

Testing procedure:

Rats normally consume 5-6 g food/100 g body weight/day (Sharp and La Regina, 1998). Prior to and during training and testing, rats were food restricted, receiving approximately 2 g food/100 g body weight/day; water intake was not restricted. Weight was monitored and rats were reduced to no more than 80% of their original body weight and were maintained at a reduced level throughout training and testing. All the rats lost weight in a similar manner, regardless of their initial weight. The average percentage of weight lost by these rats over the two-week period was 14.2%.

All animals were given shaping periods for 2-3 days in the box. During the shaping period, the Plexiglas divider was initially raised 1 inch above the floor. The divider was then lowered to a ½ inch, and finally flush against the floor. The repeated exposure to the testing apparatuses during the shaping period also served to acclimate the rats to the testing environment.

After the shaping period, rats were trained three times per day for 5 days. In order to advance to the testing phase of the experiment, animals had to perform the DFR task successfully at a rate of no less than 90% to ensure that their CST was intact and functioning preoperatively. Animals were tested twice a day for 5 days, and pre-surgical data were collected. After pre-surgical testing was completed, rats were allowed to regain weight for a two-week period before

undergoing surgery. Ten rats per group were randomly assigned to either the Sham Control (non-lesion) or the Lesion group just prior to surgery. After surgery, rats were allowed to recover for one week, and were then trained once a day, five days a week, through out the remaining recovery period. After recovering for six weeks, rats were tested twice a day for five days, and post-surgical data were collected by a blinded investigator.

The data collected included the total number of attempts and the number of successful attempts. An unsuccessful attempt was scored only when a rat reached into a slot and displaced the pellet or dropped it through the gap in the floor. This conservative method of counting attempts was used to reduce arbitrariness by the investigator and was an important consideration for interpretation of the data. A successful attempt was scored when a rat reached into a slot, grasped a pellet, lifted it over the gap in the floor, and pulled it through the Plexiglas divider to the main compartment of the testing apparatus. A one-way analysis of variance with a Bonferroni's multiple comparison post-test was performed using GraphPad Prism version 3.00 for Windows, GraphPad Software, San Diego California USA. Data is presented as mean percentage \pm standard deviation.

Surgical procedures:

After pre-surgical testing, rats were required to return to at least 95% of their original weight before undergoing surgery. Following anesthesia with isoflurane, the surgical site was shaved and the rat was placed on an operating

board designed to bend the head forward, increasing exposure of the upper cervical vertebrae and spinal cord. A skin incision was made from the base of the skull to the top of the scapulas and the muscles were retracted. A laminectomy was performed exposing the dorsum of the spinal cord over its full width between cervical level 2 and cervical level 4. In the 20 rats receiving a spinal cord lesion, the dorsal columns at cervical level 3 were identified bilaterally. A suture needle was inserted horizontally in the middle of the spinal cord, separating the dorsal and ventral aspects of the spinal cord. The suture thread was gently lifted and a pair of iridectomy scissors was used to bilaterally transect the dorsal half of the spinal cord, thereby completely lesioning the CST in the dorsal funiculus. The suture thread was then lifted through the dorsal hemisection cut to assure that a complete dorsal hemisection was achieved. A pledget of gelfoam was placed over the wound and the overlying muscles and skin were sutured. The rats were placed on heating pads to maintain body temperature and observed until fully recovered from the anesthesia. The analgesic buprenorphine (0.1 mg/kg/8 hours, S.C.) was administered for 24 hours post-surgery.

Results

Immediate post-operative observations:

All animals were observed immediately after surgery for signs of illness, lack of bladder expression, infection, or wound opening, but were not systematically analyzed for neurological deficits. A slight paresis and/or ataxia was noted in some animals, but these symptoms resolved within a week of surgery.

Results from testing conducted in the original apparatus:

Pre-surgical testing results

Twenty rats were tested in this experiment. Their pre-surgical scores (Fig. 2) demonstrated that all of the animals were able to perform the behavioral task successfully at least 90% of the time, indicating that their CST was intact. No significant difference ($p > 0.05$) in pre-surgical testing scores was found between the animals assigned to the Sham Control (95.3% \pm 4.5) and Lesioned (96.2% \pm 2.7) groups.

Post-surgical testing results

The rats in the Sham Control group showed no significant difference ($p > 0.05$) between their pre-surgical (95.3%) and post-surgical (93.4% \pm 3.8) scores (Fig. 2). However, post-surgically, the 10 rats in the Lesioned group tried to perform the behavioral task and were unsuccessful. As a result, they were unable to retrieve their food reward, and the behavior extinguished. When tested, because they did not perform the task, their testing score was 0% \pm 0.3

(Fig. 2). However, observation of the animals demonstrated that they were able to reach and grasp food dropped in their cages to some extent, leading us to believe that the post-surgical scores of the lesioned animals were misleading. To determine if the rats were truly unable to perform the behavioral task, or if the low scores observed were the result of extinguished behavior, the testing apparatus was modified as described in the Materials and Methods.

Results from testing conducted in the modified apparatus:

Pre-surgical testing results

In this experiment, twenty rats were used. The behavioral task was performed successfully at least 90% of the time by all the animals (Fig. 3), indicating that their CST was intact. As with the first experiments, no significant difference ($p>0.05$) was found in pre-surgical testing scores between the animals assigned to the Sham Control (95.6% \pm 4.6) and Lesioned (94.4% \pm 3.9) groups.

Post-surgical testing results

There was no significant difference ($p>0.05$) between the pre-surgical (95.6%) and post-surgical (94.4% \pm 4.9) scores of the Sham Control animals (Fig. 3). Unlike the rats in the Sham Control group, the rats in the Lesioned group were significantly impaired ($p<0.001$) after surgery. While the Lesioned rats performed the task successfully 94.4% of the time pre-surgically, their success rate dropped to 39.9% \pm 3.8 post-surgically.

Discussion

The goal of spinal cord research is to repair injured axons so that ultimately sensory and motor function can be restored. Advances in spinal cord injury research have resulted in numerous potential treatments to improve sensory and motor function after spinal cord injury (Li and Raisman, 1994; Joosten et al., 1995a; Kalderon and Fuks, 1996; Bregman et al., 1997a; Brosamle et al., 2000; Ramon-Cueto et al., 2000). Adequate evaluation of the efficacy of these potential treatments is essential. While the majority of spinal cord injury studies that examine CST lesions utilize a thoracic injury model (Kuhlengel et al., 1990; Fernandez et al., 1993; Schnell and Schwab, 1993; Tetzlaff et al., 1994; Joosten et al., 1995b; Kalderon and Fuks, 1996; Schwab and Brosamle, 1997; Li et al., 1998; Benowitz et al., 1999; Ramon-Cueto et al., 2000), the number of studies using a cervical lesion model to transect the CST is increasing (Kuhlengel et al., 1990; Fernandez et al., 1993; Firkins et al., 1993; Tetzlaff et al., 1994; Joosten et al., 1995b; Kalderon and Fuks, 1996; Schwab and Brosamle, 1997; Li et al., 1998; Benowitz et al., 1999; Ramon-Cueto et al., 2000). The growing use of a cervical injury model to examine CST injuries will lead to more behavioral testing of this system, which results in subtle functional deficits when damaged (Whishaw et al., 1986). Clearly, it is necessary to have a testing procedure that examines cervical CST function in normal and injured rats and results in quantitative data.

Numerous tests exist that examine general motor function, including the open field test (Wrathall, 1992a; Basso et al., 1995; von Euler et al., 1996) and

the incline plane test (Rivlin and Tator, 1977; Gale et al., 1985; Kerasidis et al., 1987). While these tests can be used to describe an animal's general motor performance, they are not able to provide information about improvements in function of a specific motor tract (Kunkel-Bagden et al., 1993). Since damage to the CST does not result in gross motor impairments in the rat, tests such as the open field and inclined plane tests are not adequate to assess injury and repair of this system. After a thoracic CST lesion, tests such as grid walking (Kunkel-Bagden and Bregman, 1990; Grill et al., 1997; Z'Graggen et al., 1998), footprint analysis (Behrmann et al., 1992; Kunkel-Bagden et al., 1993; Cheng et al., 1997), and contact placing (Kunkel-Bagden et al., 1992; Bregman et al., 1995; Palladini et al., 1996), are used to assess injury and recovery, but these tests are not useful for examining forepaw function.

The sticker removal test has been utilized to examine forelimb impairment (Schrimsher and Reier, 1992; Diener and Bregman, 1998a). However, while the sticker removal test can be employed to examine forelimb movement, it is not sufficient for analyzing varying degrees of CST impairment and improvement. In this test, a sticker is placed on the bridge of a rat's nose. Removal of the sticker requires full range of motion of the forelimbs and is facilitated if some grasping ability is intact. A rat's behavior when performing this task is rated on a scale from 0 to 6. A rat can score up to a 4 on this scale without ever attempting to grasp the sticker, and can score a 5 if the sticker is removed after numerous attempts without a strong grasp. If the sticker is tightly grasped and quickly removed, then a score of 6 is given. As five of the seven scoring measures used

to assess this task are based on forelimb movement, not grasping ability, the amount of quantitative data that can be obtained from utilizing this test to examine the range of CST integrity is limited. Therefore, this is not the ideal test for examining CST integrity of the forelimbs. The DFR task, however, can be used to examine the integrity of the CST in rodents after cervical lesions resulting in quantitative data that can be used to determine not only if the CST is damaged, but can also indicate the degree of damage or repair.

In our experiments using the original DFR apparatus, we obtained results similar to what we were expecting when the animals were trained and tested pre-surgically (Sham, 95.3%; Lesioned, 96.2%). When the rats underwent post-surgical training, the Sham Control rats were able to perform the DFR task successfully. However, those rats that were lesioned were not capable of performing the task successfully during the second week post-surgery. Because they attempted the DFR task during this time frame, but were unable to obtain the food reward, they had no incentive to continue to perform the task and, before any function returned that would allow them to do the task, the behavior extinguished.

When a new behavior is being taught, a reward is provided to encourage repetition of the behavior. In our experiments, to promote directed reaching of the forepaw, a food pellet was used as the reward for successful execution of the behavior. The behavior is subsequently maintained as long as a reward is available, at least sporadically. However, if the reward is withdrawn completely, or is unable to be retrieved because the animal cannot successfully perform the

task necessary to obtain the reward, two events will occur. The first is an extinction burst, where the behavior is repeated numerous times at an increased frequency in order to try to obtain the reward. If the repeated performance of the behavior still does not result in a reward, the second event occurs whereby the behavior stops completely, and is said to be extinguished.

This extinguishing of the learned DFR behavior during the immediate post-surgical period prevented us from obtaining reliable post-surgical scores from the lesioned rats (0%). This depression of function in the immediate post-surgical period followed by a gradual restoration of limited function has been previously reported (Price and Fowler, 1981). By modifying the shelf that the food reward was placed upon, this problem of extinction was rectified. When the animals that were unable to perform the DFR behavior were trained post-surgically in the modified DFR apparatus, the gap in the floor was closed by pushing in the shelf containing the food reward. By implementing this modification, those animals that were unable to perform the DFR task were still able to receive a reward for maintaining the reaching behavior associated with the DFR task. As the rats recovered during the two weeks post-surgery, a limited amount of grasping ability returned, and the shelf was gradually pulled back out until the gap in the floor was $\frac{3}{4}$ inch. This modification was critical for accurately assessing the rats' functional abilities, as demonstrated by the difference between the post-surgical DFR score for the Lesioned rats tested in the original apparatus (0%) and the modified apparatus (39.3%).

Other researchers have used DFR to assess CST impairments with varied outcomes. Li and colleagues (Li et al., 1997) examined the effects of ensheathing cells to promote functional recovery of the CST after a cervical lesion. In 28 rats, the CST was destroyed unilaterally by focal electrolytic lesions; of these rats, seven were treated with ensheathing cell transplants and the rest were lesion-control animals. The rats were subsequently tested to “look for the presence or absence of directed forepaw reaching function” 2-3 months after surgery. A bracelet was placed on the forepaw ipsilateral to the lesion, forcing the rats to use their contralateral, impaired forepaw. In the non-treated group, histological examination revealed that 13 rats had 20-50 % of CST fibers spared, 1 rat had 1-2 % of CST fibers spared, and 7 rats had no spared fibers. When DFR was examined in these 21 rats, all rats with any spared fibers (14 total) were found to have DFR “present”. However, in all seven control rats with no CST fibers spared after lesioning, DFR was found to be “absent”. In the group treated with ensheathing cell transplants, histological examination revealed complete unilateral destruction of the CST in all seven rats. In four of these rats, the ensheathing cells transplants were found to have formed a continuous column associated with axons that bridged the entire length of the lesion in the host CST. When DFR was tested in these animals, it was found to be “present”. In the remaining three rats, which were found to have either no ensheathing cells, or the ensheathing cells did not span the length of the lesion, DFR was reported to be “absent”. Therefore, in all rats that were found to have a completely lesioned, but unrepaired CST (7control, 3 treated), DFR was reported

to be “absent”. In fact, it was reported that these rats “never reached through the aperture with the other [impaired] paw.” The “absence” of not only grasping, but also reaching, was found in those rats that were the most severely impaired, and therefore would be unable to obtain their food reward. Since damage to the CST results in an inability to grasp, but spares reaching behavior, the “absence” of DFR in these rats was more likely to be extinction of the behavior than an inability to reach.

Säling (Saling et al., 1996) examined adaptive behavioral reactions in reaching following cortical lesions in rats. In part of the study, adult rats received discrete bilateral lesions of the motor cortex responsible for the forelimbs. Rats were trained to perform a reaching task using two different box designs. In one design, the rats were trained to reach through a grid to retrieve pellets, and in the other design, rats were required to reach into a horizontal tube to retrieve pellets. Rats were trained to perform the DFR task prior to surgery and then their performance was re-evaluated at 7, 14, 21, 28, 56, and 70 days after lesioning. The animals' performance in the grid apparatus was always better than in the tube apparatus. However, their success in both the grid and the tube apparatuses was lower on day 7 (~18% and ~1%, respectively) than on day 14 (~19% and ~5%, respectively), and lower on day 14 than on day 21 (~24% and ~12%, respectively), at which time DFR performance began to stabilize. These rats did not show an extinction of the learned behavior even though their success rate was extremely poor initially, especially in the tube apparatus. These data suggest that this retention of the DFR function was due to the time intervals used

in testing these rats. The rats were not tested daily, and therefore were not subjected to repeated failure at retrieving the food reward, which would have resulted in extinguished behavior.

Diener and Bregman, 1998a, investigated the ability of fetal spinal cord transplants to promote the development of target reaching after spinal cord injury in neonatal rats. Rats were three-days-old when they underwent an overhemisection, resulting in bilateral lesioning of the dorsal funiculus, and unilateral lesioning of the right lateral funiculus, ventral funiculus and gray matter. Histological examination of the spinal cords from the lesioned rats revealed that the CSTs in all these rats were completely destroyed bilaterally, as was the right rubrospinal tract. In the animals that received fetal spinal cord transplants, histological analysis revealed extensive transverse and rostrocaudal apposition of the transplant tissue to the damaged host tissue. As part of the study, during the sixth through ninth weeks of life, four control (non-lesioned), five lesioned, and six lesioned and treated rats were functionally evaluated. The number of rats using their forelimbs was compared to the number of rats using compensatory methods (i.e. their tongue) to retrieve a food reward. All of the control rats were found to use their forelimbs to retrieve the food reward (100%), as were most of the rats who received transplants (80-85%). In contrast, none (0%) of the rats that were lesioned but untreated used their forelimbs to retrieve the food reward. The complete lack of use of the forelimb in the rats in these experiments, however, was not a result of extinguished behavior. They were unable to perform the task because of the numerous tracts damaged in the

overhemisection, resulting in not only impairment of grasping, but impairment of reaching and other gross motor movements.

The DFR task has also been utilized by researchers to examine functional abilities in numerous other experimental models (Castro, 1972b, 1977; Price and Fowler, 1981; Whishaw et al., 1993; Whishaw and Gorny, 1996; Thallmair et al., 1998; Whishaw et al., 1998a; Whishaw et al., 1998b; Z'Graggen et al., 1998; Whishaw, 2000; Ballermann et al., 2001). Price and Fowler (Price and Fowler, 1981) used DFR to assess deficits in ipsilateral and contralateral forepaw motor control in rats following unilateral cortex ablations. The testing apparatus in this experiment was designed with two force transducer chambers outside the testing box. The transducers were designed so that a rat could only respond to Transducer I with its left paw, and to Transducer II with its right paw. Rats were trained to depress a transducer five times with a minimum force of 16 g in order to receive a water reward. After surgery, no differences were seen in either paw in the sham-operated rats. In the lesioned rats, however, the paw contralateral to the lesion exhibited permanent impairment in both the force used to strike the transducer and the rate of response. However, because the lesion was unilateral, rats were still able to perform the task appropriately with the ipsilateral limb and receive the reward, thus preventing the behavior from being extinguished.

Castro (Castro, 1977) used DFR in a study examining limb preference after lesions to one cerebral hemisphere. Sixteen adult rats were divided into two groups. The six rats in "Group A" received only post-operative DFR testing,

while the ten rats in “Group B” received both pre- and post-operative DFR testing. The testing apparatus was designed to bias the rats to use the same forepaw every time. When the rats in Group B were tested preoperatively, they did indeed use the forepaw in accordance with the testing bias. The rats in both groups underwent unilateral frontal cortical lesions. After lesioning, the rats in Group A and those in Group B all showed a preference to use the forepaw ipsilateral to the lesion (98.1% and 95.4%, respectively), even though they were tested in the apparatus that biased them to use the forepaw contralateral to the lesion. Since the rats were able to use their contralateral limb to perform the task, they were still able to retrieve the food reward and the DFR behavior did not extinguish.

As stated above, the two experimental models just described utilized unilateral lesions, resulting in one impaired and one unimpaired forepaw. Since these experiments allowed rats to use the unimpaired paw to successfully perform the DFR task and receive their reward, there was no risk that the behavior would extinguish. This unilateral model is often used when examining how a behavior is altered as a result of lesioning cortical tracts above the level of the spinal cord. When injuring cortical tracts, it is often not possible to create bilateral lesions above the level of the spinal cord without causing severe morbidity and mortality. Bilateral lesioning, however, is a more stringent model to use when examining regeneration, especially if the tract being lesioned, such as the CST, does not result in functional deficits so severe as to impair the well being of the animal. The availability of a behavioral test that can be used to

quantitatively assess forepaw function in rats with abilities ranging from normal to severely impaired after a bilateral lesion, without risk of extinguishing behavior is crucial. The DFR task with the modifications described in this paper is such a test.

The DFR task is an important tool in assessing functional recovery in rats after injury to the CST above the thoracic level of the spinal cord. The original DFR apparatus, while useful for examining other reaching and grasping related phenomena, was unable to prevent the extinguishing of the DFR behavior in rats with severe functional deficits. By modifying the original apparatus to include a movable food shelf that could be closed completely, we were able to allow the rats to maintain the reaching behavior necessary for performing the DFR task in injured rats, thereby preventing the behavior from being extinguished. Because recovery of function is the goal of patients with spinal cord injuries, much of the animal research on spinal cord injury includes behavioral tests to assess restoration of function (Bregman et al., 1995; Cheng et al., 1996; Grill et al., 1997; Guest et al., 1997a; McDonald, 1999). Many of the tests used rely on the use of a reward to elicit the desired behavior. As has been demonstrated in this paper, if the test is not designed in such a way that severely impaired animals can still obtain the food reward, then the learned behavior will extinguish. Researchers are then only able to determine if the behavior is present or absent, and are unable to examine varying degrees of restoration of function. In addition, they are unable to determine if a behavior is absent because the animal is severely impaired or because the behavior has extinguished. By designing any

apparatus/task so that it can be modified to prevent the extinguishing of a learned behavior, researchers are assured that the behavioral data they obtain accurately reflect their experimental parameters and do not result from flaws in the testing apparatus.

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Figure 1. Directed Forepaw Reaching Apparatuses

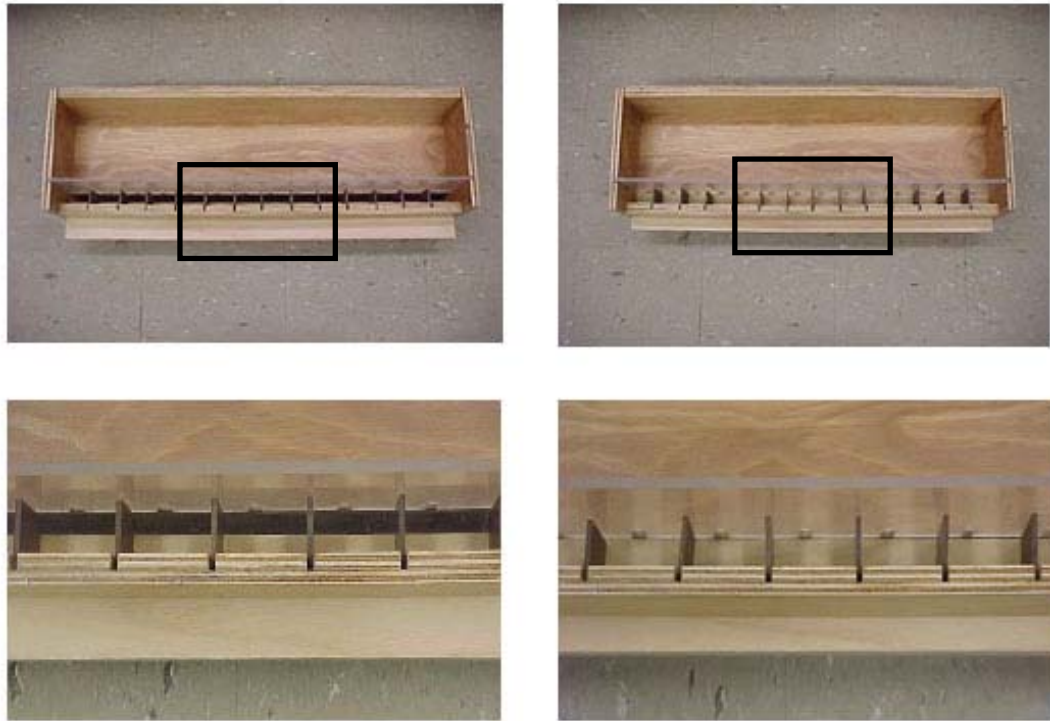


Figure 1: The original and modified DFR apparatuses, viewed from above. The entire original and modified apparatuses can be seen in panels A and B, respectively. Panel C is a close-up view of the boxed area in panel A, with the $\frac{3}{4}$ inch gap in the floor clearly visible. Panel D is a close-up view of the boxed area in panel B, in which the shelf has been closed.

Figure 2. DFR Scores in the Original Apparatus

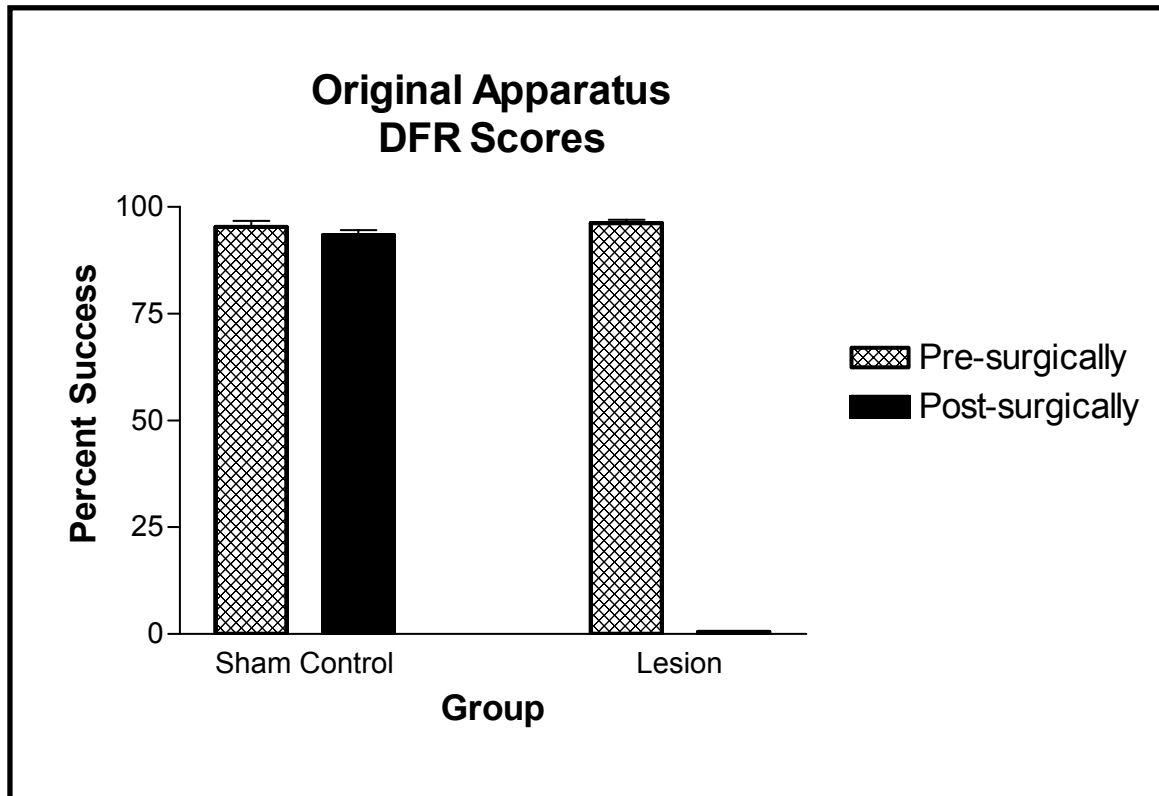


Figure 2: Successful performance of the DFR task (expressed as percentages) when tested in the original apparatus. In the pre-surgical testing period, the Sham Control rats were successful 95.3% \pm 4.5 of the trails, while the Lesioned rats performed the task successfully 96.2% \pm 2.7 of the trials. Post-surgical testing revealed that Sham Control rats were able to perform the DFR task successfully 93.4% \pm 3.8 of the trials. In contrast, the Lesioned rats performed the task with a 0% \pm 0.3 success rate.

Figure 3. DFR Scores in the Modified Apparatus

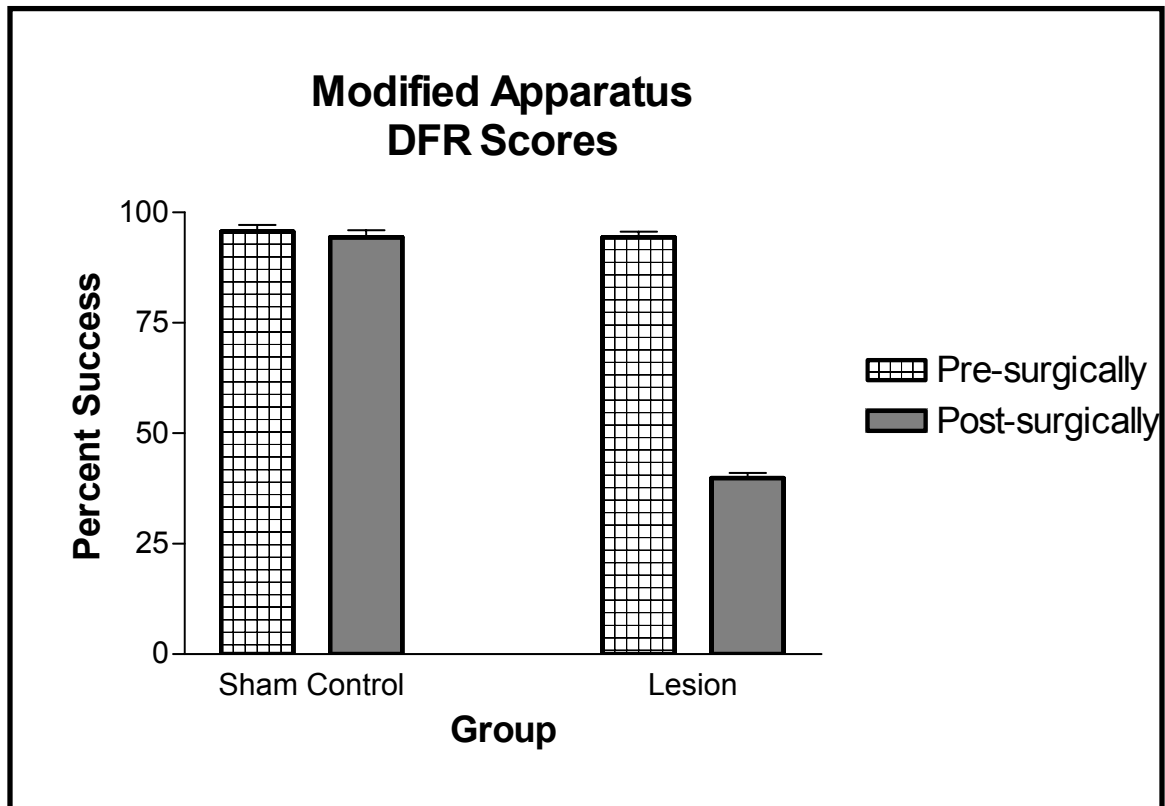


Figure 3: Successful performance of the DFR task (expressed as percentages) when tested in the modified apparatus. In the pre-surgical testing period, the Sham Control and Lesioned rats performed the task with a success rate of 95.6% \pm 4.6 and 94.4% \pm 3.9, respectively. Post-surgical testing demonstrated that the Sham Control rats performed the DFR task successfully 93.4% \pm 4.9 of the trials. The Lesioned rats performed the task successfully in 39.3% \pm 3.8 of the trials.

CHAPTER 4

Ensheathing Cells and Methylprednisolone Promote Axonal Regeneration and Functional Recovery in the Lesioned Adult Rat Spinal Cord

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Abstract

Axons fail to regenerate after spinal cord injury (SCI) in adult mammals, leading to permanent loss of function. Following SCI, ensheathing cells (ECs) promote recovery in animal models, whereas methylprednisolone (MP) promotes neurological recovery in humans. In this study, the effectiveness of combining ECs and MP after SCI was investigated for the first time. After lesioning the corticospinal tract in adult rats, ECs were transplanted into the lesion, and MP was administered for 24 hours. At six weeks post injury, functional recovery was assessed by measuring successful performance of directed forepaw reaching (DFR), expressed as percentages. Axonal regeneration was analyzed by counting the number of anterogradely labeled corticospinal axons caudal to the lesion. Lesioned control rats, receiving either no treatment or vehicle, had abortive axonal regrowth (1 mm) and poor DFR success (38% and 42%, respectively). Compared to controls, MP treated rats had significantly more axons 7 mm caudal to the lesion, and DFR performance was significantly improved (57%). Rats that received ECs in combination with MP had significantly more axons than all other lesioned rats up to 13 mm. Successful DFR performance was significantly higher in rats with EC transplants, both without (72%) and with (78%) MP, compared to other lesioned rats. These data confirm previous reports that ECs promote axonal regeneration and functional recovery after spinal cord lesions. In addition, this research provides new evidence that, when used in combination, MP and ECs improve axonal regrowth up to 13 mm caudal to the lesion at six weeks post-injury.

Introduction

Unlike axons in the peripheral nervous system (PNS), which have the capacity to regrow (Fawcett and Keynes, 1990; Raivich and Kreutzberg, 1993a), injured central nervous system (CNS) axons fail to regenerate (Schwab and Bartholdi, 1996; Nicholls et al., 1999). The one exception is found in the olfactory system. Olfactory neurons have a life span of 4 weeks (Graziadei and Monti-Graziadei, 1978); when they die, new neurons originating from neuroepithelial precursors located in the olfactory epithelium (Graziadei and Monti-Graziadei, 1978) successfully reinnervate the olfactory bulb (Kosaka et al., 1998). Fascicles of olfactory axons are ensheathed throughout the PNS (Doucette, 1991) and into the CNS (Doucette, 1993b) by a glial cell termed olfactory ensheathing cells (Doucette, 1984).

Based upon their unique ability to promote reinnervation in the olfactory system, researchers transplanted ECs after spinal cord injury (SCI) into rats to determine whether ECs retained their ability to promote reinnervation. After injection of EC suspensions at the site of a dorsal rhizotomy in rats, regenerating axons regrew into the spinal cord gray matter (Ramon-Cueto and Nieto-Sampedro, 1994). ECs transplanted into spinal cord after electrolytic destruction of the corticospinal tract (CST), resulted in the growth of CST axons through the transplant, into the host CST, and restoration of directed forepaw reaching (DFR), a measure of function (Li et al., 1997). In a subsequent study using the same experimental model, regenerating CST axons were found in parallel bundles that crossed the lesioned area and reentered the spinal cord after

transplantation of ECs into the injury site (Li et al., 1998). Using Schwann cell-filled guidance channels combined with injections of ECs, transected spinal cords were found to contain propriospinal axons that regenerated 2.5 cm (Ramon-Cueto et al., 1998). Recently, ECs were shown to promote axonal regeneration and restore climbing abilities in rats after complete spinal cord transection (Ramon-Cueto et al., 2000). Therefore, ECs are recognized as a valuable tool in promoting spinal cord regeneration (Bartolomei and Greer, 2000; Franklin and Barnett, 2000; Raisman, 2001; Treloar et al., 2001).

A common treatment for SCI in human beings is administration of methylprednisolone (MP), a synthetic glucocorticoid steroid initially developed for its enhanced anti-inflammatory activity and lessened mineralocorticoid activity relative to cortisol, the prototypical glucocorticoid (Hall, 1992). The extreme lipophilicity of MP, a drawback to its intravenous use, has been overcome by the production of a MP-hemisuccinated ester (Solu-Medrol). When Solu-Medrol is injected intravenously into the body, the ester is liberated from the pro-drug, releasing the free steroid, which is believed to cross the blood brain barrier (Hall, 1992). Although glucocorticoids have been used in clinical treatment of spinal cord trauma since the 1960's, it was animal research investigating the neuroprotective pharmacology of MP at various dosing regimes after SCI (Braughler and Hall, 1982a; McGinley et al., 1982; Braughler and Hall, 1983, 1984; Hall et al., 1984; Braughler et al., 1987) that lead to the first (Bracken et al., 1984), second (Bracken et al., 1990), and third (Bracken et al., 1997) National

Acute Spinal Cord Injury Study, to optimize parameters for neurological recovery after acute SCI in humans.

While many therapies to promote regeneration after SCI have been investigated, at present it does not appear that any single therapy will solve all the problems associated with the lack of regeneration after SCI. Two therapies shown to promote repair after SCI when used alone are EC transplants and MP injections. Therefore, we examined the effects of combining MP administration with EC transplantation, on regeneration of the CST after injury. Our results indicate that this combination of treatments promotes long-distance regrowth of CST axons and functional recovery after acute cervical SCI in the adult rat.

Materials and Methods

Subjects

Eighty-one adult Sprague Dawley rats (300-400 g, Taconic Farms, Germantown, NY) were used in this study. Twenty rats were used to harvest ECs for transplants, and 61 rats were randomly placed into control and experimental groups. Of those 61 rats, 17 were eliminated from the study either because they did not survive beyond 14 days post-surgery ($n = 15$), or, they failed to train in response to a food reward prior to surgery ($n = 2$).

Surgeries

Cultures

ECs were harvested and purified following a previously described technique (Nash et al., 2001). Briefly, the rats were anesthetized, decapitated, and the nasal and frontal bones were removed. Olfactory nerve rootlets and olfactory bulbs were detached. The rootlets and outer two layers of the olfactory bulb were dissected, retained, and then triturated and trypsinized. After the cells were centrifuged, media was added to yield a suspension containing 8.5×10^5 cells/ml. The cell suspension was seeded into an uncoated flask and incubated (48 hr, 37°C and 5% CO₂) for purification. The purified ECs (93%), which remained in the supernatant, were centrifuged and labeled with Cell Tracker[®] green (50 μ M, Molecular Probes, Eugene, OR) immediately prior to transplantation.

Surgical procedures

Rats were randomly assigned to control or experimental groups. The three control groups were: Sham (n = 5), Lesion (n = 5), and Vehicle (n = 5); and the three experimental groups were: MP (n = 10), EC (n = 9) and MP/EC (n = 10). A summary of the groups in this experiment and the treatment (s) each group received is found in Table 1.

Following anesthesia with isoflurane (5%, Abbott Laboratories, North Chicago, IL) and application of ophthalmic ointment (E. Fougera & Co., Melville, NY), the rats were placed on an operating board in such a way as to bend the cervical spinal cord. A laminectomy was performed exposing the dorsum of the spinal cord between C2 and C4. The dorsal columns were identified bilaterally and, in all rats except for those in the Sham group, a suture needle was passed through the spinal cord, isolating the dorsal funiculus. The suture thread was gently lifted and a pair of iridectomy scissors was used to bilaterally transect the dorsal funiculus, thereby transecting the CST. Visualization of the dorsal horns and the central gray commissure confirmed accuracy of the lesion borders. ECs pre-labeled with Cell Tracker™ green were injected (10 µl Hamilton syringe) into the cut surfaces of the CST (two injections in the rostral surface and two in the caudal surface). Each injection site received 0.5 µl of a suspension containing 50,000 ECs in Dulbecco's Minimum Essential Media (DMEM, Biofluids, Rockville, MD). Rats receiving the vehicle for ECs were injected with an equal volume of DMEM into the CST. A pledget of biodegradable gelfoam soaked in fluorogold (3% in 0.9% saline, Molecular Probes) was placed in the lesion site (See Tracers

below). The overlying muscles and skin were sutured, and the rats were placed on a heating pad to maintain body temperature. Each rat received a single dose of buprenorphine (0.1 mg/kg, Reckitt & Colman Pharmaceuticals, Richmond, VA) immediately after surgery to alleviate pain. One hour after the spinal cord was lesioned, a bolus injection of MP sodium succinate (Solu-Medrol, 30 mg/kg, Pharmacia Upjohn, Kalamazoo, MI) in 0.9% saline was administered in a tail vein. This injection was followed by four subsequent administrations of MP into a tail vein (30 mg/kg) at six-hour intervals. Rats receiving vehicle for MP underwent the same treatment regime, but were injected with an equal volume of 0.09% saline in a tail vein. For all lesioned rats, their bladders were manually expressed immediately after surgery and three times a day until spontaneous urination returned.

Tracers

During surgery (see Surgical Procedures), a fluorescent retrograde tracer, fluorogold, was administered to identify the neurons whose axons were transected. Seven weeks post-injury, rats were prepared for injection of biotin dextran tetramethylrhodamine (BDT, Molecular Probes). This fluorescent anterograde tracer, injected into the primary motor cortex, was used to label CST axons caudal to the lesion site in the spinal cord. Following anesthesia with isoflurane (5%), rats were placed in a stereotaxic instrument and a total of six stereotaxically determined holes (0.9 mm diameter) were drilled in the skull over the primary motor cortices associated with the forelimbs. The anterior/posterior (A/P) and medial/lateral (M/L) coordinates for these injections, from bregma,

were: +0.5 A/P, ± 3.5 M/L; +1.5 A/P, ± 2.5 M/L; and +2.5 A/P, ± 1.5 M/L. All injections were delivered at a depth of 2.5 mm from the surface of the skull. A 10 μ l Hamilton syringe was used to inject BDT bilaterally into layer V of the cortex. Three injections into each cortical hemisphere were utilized to administer a total of 1.2 μ l of the anterograde tracer. Bone wax (Ethicon, Somerville, NJ) was used to seal the holes in the skull, the scalp was sutured, and a single dose of buprenorphine (0.1 mg/kg) was administered immediately after surgery to alleviate pain. Rats were euthanized three days after tracer injections.

Functional Testing

Apparatus

A modified version of an apparatus (Castro, 1972b) designed to test directed forepaw reaching (DFR) was used to measure grasping ability (Figure 1A & 1B). The apparatus was a box with two compartments separated by a Plexiglas divider. The two compartments were comprised of a main compartment (30 $\frac{1}{2}$ x 6 $\frac{1}{4}$ x 5 inches) for the rat and a minor compartment (30 $\frac{1}{2}$ x 3 $\frac{1}{2}$ x 3 inches) for the food reward, which was subdivided into 14 adjacent slots of equal size (2 x 1 $\frac{1}{4}$ x 3 inches). The floor of the minor compartment was made of a movable shelf. The shelf was usually pulled back to produce a $\frac{3}{4}$ inch opening in the floor between the compartments (Figure 1A), but could be pushed in (see Testing procedures) to produce a continuous floor between the two compartments (Figure 1B). All pre- and post-surgical testing was performed with the $\frac{3}{4}$ inch opening in the floor. The Plexiglas divider (30 $\frac{3}{4}$ x $\frac{1}{4}$ x 5 inches)

separating the rat compartment from the food compartment had 14 semicircular holes drilled into the bottom, each of which was $\frac{1}{2}$ inch in diameter.

A single 190 mg food pellet (Bio-Serv, Frenchtown, NJ) was placed into each of the 14 slots in the minor compartment. The rats were required to extend a forelimb through the holes in the Plexiglas divider in order to grasp a food pellet. The rats could use either forelimb to retrieve the food pellet, but the holes in the Plexiglas divider were only large enough to admit one limb at a time. The opening in the floor ensured that the rats grasped and lifted the food pellet in order to retrieve it. If a rat attempted to scoop or rake a pellet out of the slot without grasping and lifting it, the pellet would fall irretrievably through the opening in the floor.

Testing procedures

Pre-surgery. Rats were food restricted, receiving approximately 3 g food/100 g body weight/day, prior to and throughout training and testing. Weight was monitored to ensure rats were reduced to no less than 80% of their original body weight at any time. All rats were given shaping periods for 2-3 days in the box, to allow them to learn the task while they became familiar with the testing situation. During these shaping periods, the Plexiglas divider was initially raised 1 inch above the floor, then lowered to a $\frac{1}{2}$ inch, and then positioned flush against the floor; all rats were trained and tested at this most stringent level. Animals were trained twice a day for 5 days then tested twice a day for 5 days and pre-surgical DFR data were collected. Rats were required to return to at

least 95% of their original weight, to ensure that they were healthy, before undergoing surgery.

Post-surgery. Rats were trained twice a week during weeks 2-5 post-surgery. Some rats were profoundly impaired when they attempted to perform the grasping portion of the DFR task in the early post-surgical period, and were therefore completely unable to retrieve the food reward. For those rats, the opening in the floor of the apparatus was closed (Figure 1B) to allow the food to be raked into the main compartment, ensuring that the reaching portion of the DFR task did not extinguish. The severity of the grasping impairment decreased as the post-surgical period increased, and the opening in the floor was gradually re-established. By the end of the post-surgical recovery period, all rats were able to successfully perform the DFR task, to some degree, with the $\frac{3}{4}$ inch opening in the floor of the apparatus (Figure 1A). During the sixth week post-surgery, rats were tested twice a day for five days and post-surgical DFR data were collected by a blinded investigator.

The data were collected in terms of total number of attempts and percentage of successful attempts. An attempt was scored only when a rat reached into a slot and displaced the pellet or dropped it through the gap in the floor. A successful attempt was scored when a rat grasped a pellet, lifted it over the gap in the floor, and pulled it through the Plexiglas divider so the pellet was in the main portion of the testing apparatus. This conservative method of counting was used to reduce bias by the investigator and was important for interpretation of the data. Data were analyzed using the statistical software program Prism. A

one-way analysis of variance was performed, with an alpha level of 0.05, followed by a Bonferroni's multiple comparison test. Data are presented as mean percentages \pm standard error of the means.

Histology

Seven weeks and three days after lesioning, rats were anesthetized with chloral hydrate (10 ml/kg, Sigma, St. Louis, MO) and perfused transcardially with 300 ml phosphate buffered saline (pH 7.4) followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer. After euthanasia, all brains and spinal cords were removed and soaked overnight in 30% sucrose in a 0.1 M phosphate buffer solution. The brains were cut coronally and the spinal cords were cut horizontally, at a thickness of 20 μ m with a freezing microtome and mounted on ProbeOn™ (Fisher Scientific, Pittsburgh, PA) coated slides. Brain and spinal cord sections were examined using a Nikon Labophot fluorescent microscope and images were captured using a Sony DKC 5000 Catseye digital still camera. Detection of fluorogold-labeled neurons in layer V of the forelimb representation of the primary motor cortex confirmed lesioning of the CST, as fluorogold is taken up by cut axons, but not intact axons.

Axonal Counts

The spinal cord caudal to the lesion was examined and the BDT-labeled axons occupying the region of the spinal cord normally occupied by the CST were counted. After consulting with a biomedical statistician, it was determined

that with a 95% confidence level, and a standard deviation of 10 axons, a sampling fraction of 1:4 sections would result in a precision of $X \pm 5.4$ axons. A random starting section to begin counts was determined by randomly selecting a number between 1 and 4. For each section, the number of BDT-labeled axons was counted at 3 mm intervals caudal to the lesion, beginning 1 mm distal to the injury (i.e. 1 mm, 4 mm, 7 mm, etc.) and ending 19 mm caudal to the lesion site. Innervation of the rat forepaw extends to T1, a distance of 15.1 mm from the lesion at C3. Therefore, analysis of the axons out to 19 mm caudal to the lesion ensured that the entire distance representing the forepaw was examined. At each interval, the total number of BDT-labeled axons (left and right CST combined) along a 500 μ m length (length of microscope field) was counted. In each field counted, the focal plane was adjusted up and down to ensure that a single continuous axon was not double counted if it traversed out of the focal plane and reemerged further down in the same field. After the data were compiled, they were analyzed using the statistical software program Prism. A one-way analysis of variance was performed, with an alpha level of 0.05, followed by a Bonferroni's multiple comparison test for the data from all the groups at each interval counted. Data are presented as mean number of axons \pm standard error of the means.

Results

Immediate post-operative observations

During the week after surgery, all animals were observed for lack of bladder control, wound opening, and, signs of infection, but were not systematically analyzed for neurological deficits. In some animals, a slight paresis and/or ataxia was noted, but these symptoms resolved within two weeks of surgery.

Functional Recovery

Rats were tested prior to surgery to establish a DFR pre-surgery baseline. This pre-surgical baseline ensured that all rats were functionally capable of performing the DFR task prior to lesioning. To prevent differences in pre-surgical ability from influencing the post-surgical scores, all rats were required to perform the DFR task at a success rate of at least 90%. As noted at the beginning of the Materials and Methods, two rats did not perform at this level, and were therefore eliminated from the study. All other rats met the criterion necessary for inclusion in the study. The pre-surgery DFR scores (Figure 1C) for each group were:

Sham = 95.95 ± 3.91 , Lesion = 95.19 ± 3.72 , Vehicle = 95.41 ± 4.11 , MP = 95.20 ± 4.59 , EC = 94.40 ± 3.47 , and MP/EC = 93.97 ± 3.89 .

During the sixth week post-injury, rats were tested to assess their ability to perform the DFR task (Figure 1D). Using this functional test, the Sham animals performed the DFR task as well post-surgically ($94.86\% \pm 3.86$) as they did pre-surgically. This finding demonstrated that only the lesion, and no other portion of

the surgical procedure, inhibited the rats' abilities to perform the DFR task.

When compared to the Sham group, a significant functional deficit was noted in all rats that were lesioned, regardless of their treatment group. However, this functional deficit in performing the DFR task was not the same across all the experimental groups.

The Lesion and Vehicle groups were the most impaired of all the groups after surgery. The Lesion and Vehicle groups were able to perform the DFR task with a success rate of only $38.38\% \pm 8.31$ and $42.41\% \pm 7.10$, respectively; there was no significant difference between these two groups. The MP group, with a success rate of $56.66\% \pm 5.63$, performed significantly better than the Lesion and Vehicle groups. The EC group performed the DFR task significantly better than the Lesion, Vehicle, and MP groups, with a success rate of $71.84\% \pm 5.20$. Like the EC group, the MP/EC group, with a success rate of $78.26\% \pm 6.68$, performed the DFR task significantly better than the Lesion, Vehicle, and MP groups. Although the group that received the combination treatment of ECs and MP performed the DFR task with a higher success rate (78.26%) than the group that received ECs alone (71.84%), this difference was not statistically significant.

Histology and Axonal Counts

Fluorogold Labeled Neurons

The primary motor cortex was examined in all rats used in this study. All of the brains contained fluorogold-labeled neurons in layer V of the primary motor cortex, confirming that these CST axons were transected during the lesioning

procedure (Figure 2). Because all CST axons were transected during surgery, and not just those in the forelimb representation, fluorogold-labeled neurons were found throughout the primary motor cortex in layer V. The only exception to this labeling pattern was in the brains of the rats that were in the Sham group, whose brains had no fluorogold label.

Biotin Dextran Tetramethylrhodamine-Labeled Axons

All of the groups showed some CST axonal growth caudal to the lesion, although regrowth was not necessarily found in all of the animals in every group. There were three rats (two Lesion, one Vehicle) that did not have any BDT-labeled CST axons caudal to the lesion site. To ensure that the lack of labeled CST axons did not result from a problem with the BDT or the labeling technique, the spinal cords of these three rats, were examined rostral to the lesion. All three were found to have BDT-labeled CST axons proximal to the injury site. In all groups, CST axons were found to course in the white matter of the dorsal funiculus, and on occasion were seen to enter the gray matter. When these CST axons did migrate into the gray matter, they coursed along the border between the gray and white matter.

The number of BDT-labeled axons present was examined for all control and experimental groups at 1 mm, 4 mm, 7 mm, 10 mm, 13 mm, 16 mm, and 19 mm caudal to the lesion site. Presented in Table 2 is the mean number of labeled axons for each group at each distance examined. A graph comparing the means from the groups and a representative photomicrograph from each group, are presented for 1 mm, 7 mm, 13 mm, and 19 mm (Figures 3 – 6,

respectively). Differences between the means of groups were difficult to visualize on most of the graphs when the Sham data was included because the means for the Sham group were much larger than the means of all the other groups, except at 1 mm. Therefore, the data for the Sham group are only included in Table 2 and Figure 3, which presents the data for the groups at 1 mm caudal to the lesion. Throughout all the examined intervals, the mean number of axons were highest in the Sham group (Table 2), and at each distance examined, the mean number of labeled axons in the Sham group was significantly higher than in all other groups, with one exception, which is noted below.

Supporting the functional data reported above, there was no significant difference between the means of the Lesion and Vehicle groups at any distance examined (Figure 3A – 6A). In these two groups, axons were found only a short distance caudal to the injury, and by 7 mm distal to the lesion, all the tissue was virtually devoid of axons in both the Lesion (Table 2, Figure 3B – 6B) and Vehicle (Table 2, Figure 3C – 6C) groups.

In the MP group, axons could be found through 7 mm (Table 2, Figure 3D and 4D). At 13 mm and beyond, however, the MP had very few labeled axons (Table 2, 5D and 6D). When analyzed statistically, the MP group had significantly more axons than the Lesion and Vehicle groups at 1 mm, 4 mm, and 7 mm (Table 2). However, at 10mm, 13mm, 16 mm and 19 mm, no significant differences were found among the means of these three groups (Table 2).

In examining the photomicrographs of the tissue from the EC group (Figure 3E – 6E), it can be seen that this group had many more labeled axons than the Lesion (Figure 3B – 6B), Vehicle (Figure 3C – 6C), or MP (Figure 3D – 6D) groups. Although the number of labeled axons decreases proportionally to the distance caudal to the injury (Table 2), axons were visible even at the furthest distance analyzed (Figure 6E). In all tissue examined in the EC group, Cell Tracker™ green-labeled ECs were found in the areas where regrowing axons were located (Figure 7). Analysis of the data demonstrated that, compared to the Lesion, Vehicle, and MP groups, the EC group had significantly more axons at all distances examined (Table 2, Figure 3A – 6A). These data support the functional findings, which demonstrated that the rats receiving ECs regained significantly more CST function than the Lesion, Vehicle, and MP groups.

At 1 mm distal to the injury, the mean of the MP/EC group was not significantly different from the rats in the Sham control group (Table 2, Figure 3A). After 1 mm, the Sham group had significantly more axons than the MP/EC group (Table 2). As with the EC group, the number of labeled axons in the MP/EC group decreased as distance from the lesion increased (Table 2, Figure 3F – 6F), but axons could still be seen at 19 mm (Figure 6F). As in the EC group, the MP/EC group exhibited ECs that were visible throughout the regions where axons were found (Figure 7). Statistical analysis revealed that the MP/EC group had significantly more axons than the Lesion, Vehicle, and MP groups at all distances examined (Table 2, Figure 3A – 6A), which agrees with the functional findings presented above. When the means from the group that

received the combined treatment were compared to the means from the group that received only ECs, significant differences in results occurred only at certain distances caudal to the injury (Table 2). Analysis of the data from 1, 4, 7, 10, and 13 mm demonstrated that the MP/EC group had significantly more axons than the EC group (Table 2, Figure 3A – 5A). However, no significant differences between the means of these two groups were found at 16 mm and 19 mm caudal to the lesion (Table 2). This lack of significant differences between the means of the EC and MP/EC groups at the furthest distances analyzed may explain the lack of a significant difference between the DFR performance of these two groups.

Discussion

Currently, there is no treatment regime available to restore sensory and motor function after debilitating SCI in humans. Yet progress is being made in animal research. Numerous treatment strategies are being investigated to repair damaged axons after SCI in rats (David and Aguayo, 1981; Goldberg and Bernstein, 1987; Schnell and Schwab, 1993; Joosten et al., 1995a; Xu et al., 1995a; Chen et al., 1996; Kalderon and Fuks, 1996; Bregman et al., 1997a; Guest et al., 1997a; Oudega et al., 1997; Ye and Houle, 1997; Ramon-Cueto et al., 1998). Among these strategies are the transplantation of various cell types, including Schwann cells (Kuhlengel et al., 1990; Li and Raisman, 1994; Xu et al., 1995a; Chen et al., 1996; Martin et al., 1996; Guest et al., 1997a) and fetal spinal cord cells (Kunkel-Bagden and Bregman, 1990; Bernstein-Goral and Bregman, 1997; Mori et al., 1997; Bregman et al., 1998; Diener and Bregman, 1998a; McDonald, 1999). A cell type recently shown to promote regeneration after SCI is the EC. Investigators have examined the ability of ECs to promote axonal regeneration after SCI both alone (Li et al., 1997, 1998; Imaizumi et al., 2000; Ramon-Cueto et al., 2000; Lu et al., 2001) and in combination with other therapies (Ramon-Cueto et al., 1998), but no one had investigated ECs in combination with MP. The combined effects of these two therapies are critical to determine whether ECs are to be considered for use in humans, as MP is the only current treatment used clinically after an acute SCI (Bracken et al., 1984; Bracken et al., 1990; Bracken et al., 1997).

In the experiments described herein, MP was found to promote axonal regeneration and improve functional recovery after SCI when compared to untreated rats. These improvements, while statistically significant, were not sufficient to result in complete restoration of DFR function (57%), probably resulting from the limited distance that axons were found to regenerate (~ 7 mm). In contrast, treatment with ECs resulted in axonal elongation to the furthest distance analyzed caudal to the injury site (19 mm) and the highest functional scores of all the lesioned rats. This elongation was found whether ECs were applied alone or in combination with MP. Although more axons were found in the MP/EC group than the EC group at distances up to 13 mm caudal to the lesion (Table 2), this increase in axons was not enough to significantly improve DFR performance of the MP/EC group (78%) when compared to the EC group (72%). However, given more time, it is possible that the larger number of axons seen at shorter distances could extend further, resulting in more functional recovery in the MP/EC group.

It is equally critical to note is that when MP was used in combination with ECs, there was not a *decrease* in axonal regeneration or functional recovery, which was a potential concern. Astrocytes have the capacity to act as immune cells (Dong and Benveniste, 2001), and MP is known to suppress the function of immune cells (Wahl et al., 1975; Almawi et al., 1991). Since ECs share phenotypic characteristics with astrocytes, it was possible that MP could have suppressed the function of ECs, resulting in a decreased effect in the MP/EC group. Investigation into the mechanisms of action of MP and ECs yield little

insight since, as seen below, the mechanism of action for MP is still hotly debated and the mechanism of action for ECs is just starting to be elucidated.

One mechanism to explain MP's action is through binding of the glucocorticoid receptor, which mediates its anti-inflammatory properties. MP inhibits inflammatory processes induced after SCI (Hsu and Dimitrijevic, 1990) such as chemotaxis (Espersen et al., 1989) and the release of lysosomal enzymes (Schleimer et al., 1989). MP also inhibits phospholipase A2 activity (Hirata et al., 1980), which reduces the production of metabolites of the arachidonic acid cascade (Becker and Grasso, 1985) thereby reducing the formation of free radicals (Hirata et al., 1980; Becker and Grasso, 1985; Kontos et al., 1985; Williams and Higgs, 1988). MP also reduces expression of tumor necrosis factor alpha (TNF- α) decreasing the activation of NF-kB (Xu et al., 1998), thereby diminishing the intensity and duration of the inflammatory response (Xu et al., 1998). Whereas these effects are achieved with administration of MP at a dose of 0.03 mg/kg (Young et al., 1988), to be clinically effective after SCI, MP must be administered at the large dose of 30 mg/kg (Hall et al., 1984). At this dose, MP exhibits antioxidant effects, which allows MP to scavenge free radicals, thus decreasing the oxygen radical-induced lipid peroxidation of cell membranes (Demopoulos et al., 1980). This mechanism is believed to be independent of MP's glucocorticoid receptor-mediated actions, as evidenced by the large dose required to achieve these effects (Hall et al., 1984). Also supporting this theory are data demonstrating that synthetic steroids created

to have greater antioxidant effects than MP, but lacking any glucocorticoid activity, are as effective as MP when applied after SCI (Hall, 1993).

The mechanism of action of ECs is still unknown, but clues about the mechanism of action are beginning to emerge. ECs were found to promote the growth of retinal ganglion neurites in co-culture (Sonigra et al., 1999).

Speculating that calcium-mediated signal transduction may be involved in the growth promoting properties of ECs, the calcium channel inhibitors, verapamil and ω -conotoxin, as well as the BAPTA/AM, a calcium chelator, were applied to the co-cultures. Application of these inhibitors blocked the increase in axonal growth in the co-cultures, leading to the conclusion that ECs may promote regeneration through calcium-signaling pathways. A subsequent study (Kafitz and Greer, 1999) demonstrated olfactory receptor cell extension in culture was facilitated by exposure to media conditioned by ECs, indicating that the neurite growth promoting properties of ECs were, at least in part, mediated by diffusible factors. Data supporting this idea was reported earlier this year, when cultured ECs were found to express nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and glial cell-line derived neurotrophic factor (GDNF) (Woodhall et al., 2001).

Whereas these data provided two mechanisms of action for MP and insights into possible mechanisms for ECs, what they did not provide is any insight into the effects of using MP and ECs in combination. However, the study described in this paper does. These experiments demonstrated that ECs, not only alone, but also in combination with MP, are able to promote regrowth and

extension of CST axons after trauma to the adult rat spinal cord. In fact, although no statistical difference was found between the EC and MP/EC groups functionally, a significant increase in axon numbers up to 13 mm caudal to the lesion indicate that MP may improve the regenerative properties of ECs.

Results using ECs to repair damaged spinal cord axons in animal studies have been so successful that researchers are now beginning to investigate the feasibility of transplanting ECs into human beings. Pig ECs had been genetically engineered to express a human complement inhibitory protein, which is intended to decrease rejection of the tissue if transplanted into human beings (Imaizumi et al., 2000). After transplantation into adult rats after dorsal column transection, pig EC transplants promoted axonal regeneration, elongation, and remyelination, and restored impulse conduction across the lesion site (Imaizumi et al., 2000). Although rodent and porcine ECs promote repair after SCI, little has been determined about human ECs. Recently, ECs, obtained from adult human olfactory nerves removed from patients undergoing olfactory nerve resection, were transplanted into the demyelinated spinal cords of adult rats (Kato et al., 2000). Extensive remyelination, characteristic of ECs, was observed throughout the lesion site, demonstrating that human ECs are able to promote remyelination (Kato et al., 2000). These results further demonstrate that ECs have good potential for treating spinal cord injuries in human beings.

The experiments described herein confirm that ECs promote axonal regeneration and elongation resulting in recovery of DFR behavior after SCI in adult rats. The morphological and functional regenerative properties of ECs were

not hindered when used in combination with MP. Instead, the ability of ECs to promote axonal regeneration was enhanced as far as 13 mm caudal to the lesion. Demonstrating that ECs do not lose their regenerative properties when used in combination with MP provides another clinically relevant step towards solving the spinal cord injury problem.

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Table 1.

Group	N	Surgery	Treatment
Sham	5	Laminectomy	None
Lesion	5	Dorsal hemisection	None
Vehicle	5	Dorsal hemisection	DMEM injected into the spinal cord, followed by saline injected into the tail vein
MP	10	Dorsal hemisection	DMEM injected into the spinal cord, followed by methylprednisolone in saline injected into the tail vein
EC	9	Dorsal hemisection	Ensheathing cells in DMEM injected into the spinal cord, followed by saline injected into the tail vein
MP/EC	10	Dorsal hemisection	Ensheathing cells in DMEM injected into the spinal cord, followed by methylprednisolone in saline injected into the tail vein

Table 1. This table includes the six different groups used in this study, a description of the surgical procedure performed on the rats in each group, and the treatment(s) they received.

Table 2.

	Sham	Lesion	Vehicle	MP	EC	MP/EC
1 mm	59.02 ± 4.01	9.58 ± 5.53	10.95 ± 3.25	32.01 ± 3.41	43.57 ± 3.05	55.99 ± 2.96
4 mm	52.04 ± 3.65	4.72 ± 3.10	3.44 ± 2.15	21.26 ± 1.96	30.20 ± 3.65	43.04 ± 2.79
7 mm	55.22 ± 3.76	1.84 ± 1.51	1.46 ± 1.32	11.98 ± 2.04	20.22 ± 2.03	32.04 ± 3.64
10 mm	53.26 ± 3.88	0.66 ± 0.59	0.73 ± 0.73	5.85 ± 0.91	12.35 ± 2.57	22.48 ± 6.00
13 mm	54.56 ± 4.15	0.16 ± 0.14	0.38 ± 0.38	1.60 ± 0.36	5.69 ± 0.68	8.00 ± 0.25
16 mm	53.98 ± 3.26	0.80 ± 0.05	0.40 ± 0.40	0.48 ± 0.07	2.24 ± 0.49	3.73 ± 0.24
19 mm	52.19 ± 2.96	0.02 ± 0.02	0.04 ± 0.04	0.18 ± 0.03	2.18 ± 0.16	2.23 ± 0.20

Table 2. This table includes the complete set of data for the axon counts at the seven distances (1 mm, 4 mm, 7 mm, 10 mm, 13 mm, 16 mm, 19 mm) caudal to the lesion for the three control and three experimental groups used in this study. Data are presented as mean number of axons ± standard error of the mean.

Figure 1. DFR Apparatuses and Functional Performance

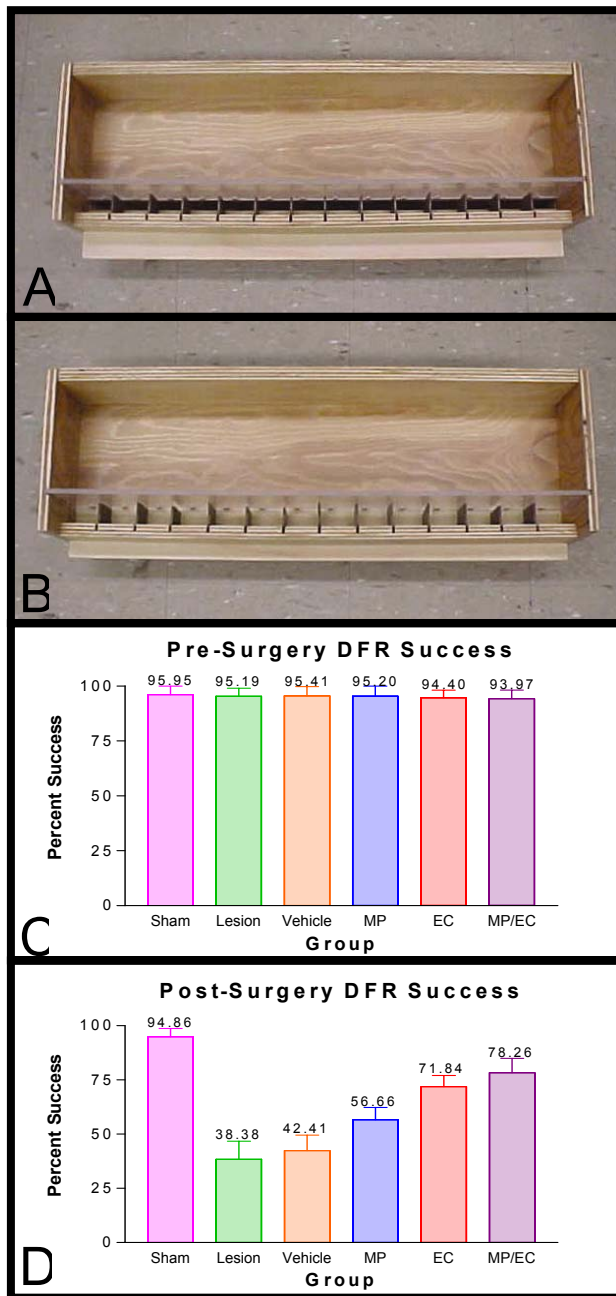


Figure 1: (A) The DFR apparatus, viewed from above, with the opening in the floor at $\frac{3}{4}$ inch, as was utilized for all testing procedures. (B) The DFR apparatus, viewed from above, with the shelf in the floor closed, resulting in no opening in the floor. This set-up was only used for animals that were unable to perform the DFR task when initially trained post-surgery. As functional recovery improved over time, the shelf was slid out again to create the $\frac{3}{4}$ inch opening in the floor. (C) Graph showing the successful performance of the DFR task (expressed as percentages of total attempts) when tested before surgery. As was required for inclusion in the study, the rats in all the groups performed the DFR task successfully at least 90% of the trials. (D) Graph showing DFR success for each group post-surgery (expressed as percentages of total attempts). The Sham group ($94.86\% \pm 0.86$) performed significantly better than all other groups. There was no significant difference in DFR success between the Lesion ($38.38\% \pm 8.31$) and Vehicle ($42.41\% \pm 7.10$) groups, although both performed significantly worse than the MP group ($56.66\% \pm 5.63$). Groups receiving ECs, both alone (EC, $71.84\% \pm 5.20$) and in combination with MP (MP/EC, $78.26\% \pm 0.668$) performed the DFR task significantly better than all other lesioned animals; however, these two groups were not significantly different from each other.

Figure 2. Fluorogold-Labeled CST Neurons

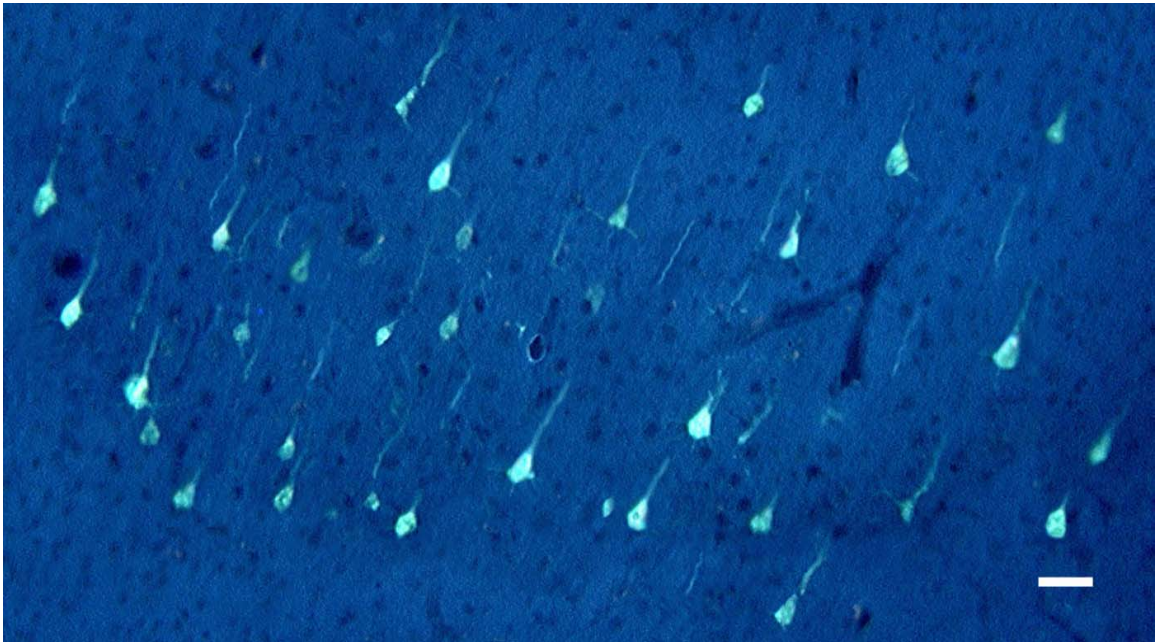


Figure 2: These neurons, filled with the retrograde tracer fluorogold, are the origin of the corticospinal axons that were severed in the spinal cord at C3. Fluorogold can be seen filling the soma, axons, and dendrites of these neurons. Scale bar = 50 μm .

Figures 3 – 6: Data from 1 mm, 7 mm, 13 mm and 19 mm caudal to the lesion. Graph of the mean number of axons (A) for each group at 1 mm (Figure 3), 7 mm (Figure 4), 13 mm (Figure 5), and 19 mm (Figure 6) distal to the injury. Representative photomicrographs of axons from the Lesion (B); Vehicle (C); MP (D); EC (E); and MP/EC (F) groups at each of these four distances caudal to the lesion. For Figures 3 – 6, axons are red because they are labeled with BDT. The Sham group data, included in both the graph (A) and the photomicrographs (G) of Figure 3, are consistent at all other distances analyzed, and therefore are not shown in the other Figures. For all photomicrographs, the scale bar = 50 μ m.

Figure 3. Data From 1 mm Caudal To The Lesion

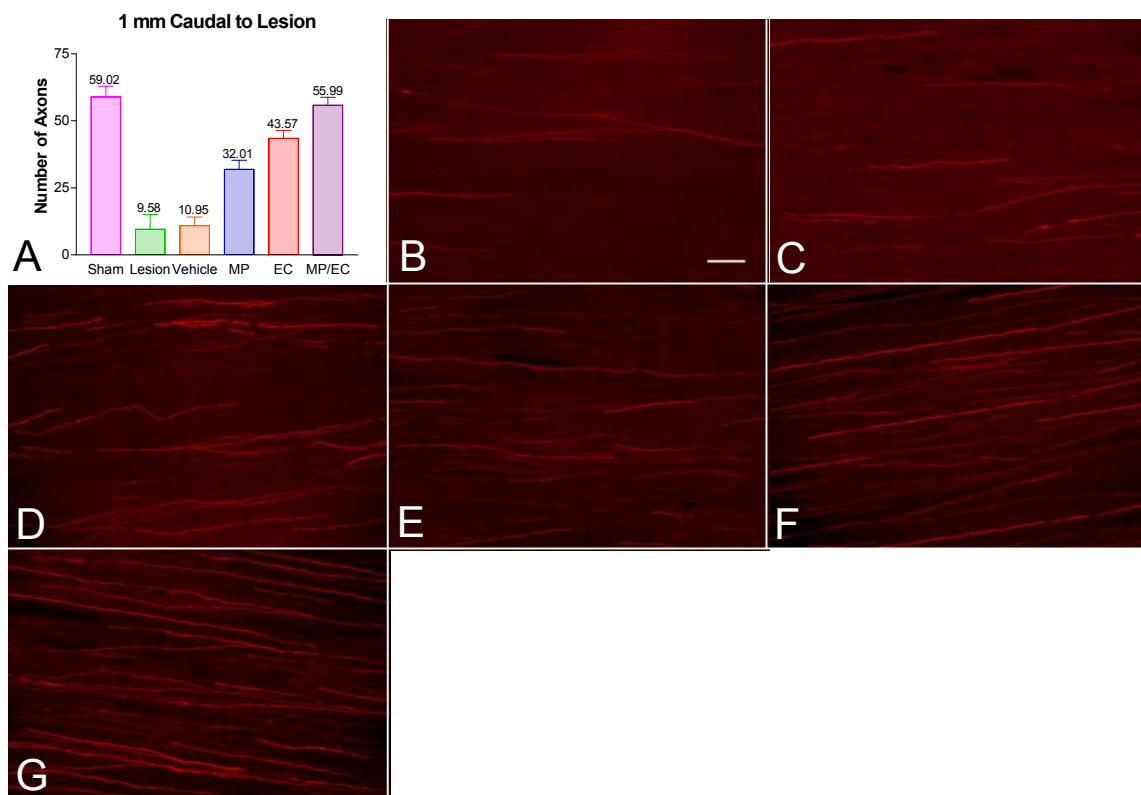


Figure 3: At this distance, there is no significant difference between mean number of axons of the Lesion (B, 9.58 ± 5.53) and Vehicle (C, 10.95 ± 3.25) groups, but there are significantly more axons in the MP group (D, 32.01 ± 3.41). The EC group (E, 43.57 ± 3.05) has significantly more axons than the MP group, and the MP/EC group (F, 55.99 ± 2.96) has significantly more axons than all other lesioned groups. At this distance only, there is no significant difference between the MP/EC and Sham (G, 59.02 ± 4.01) groups.

Figure 4. Data From 7 mm Caudal To The Lesion

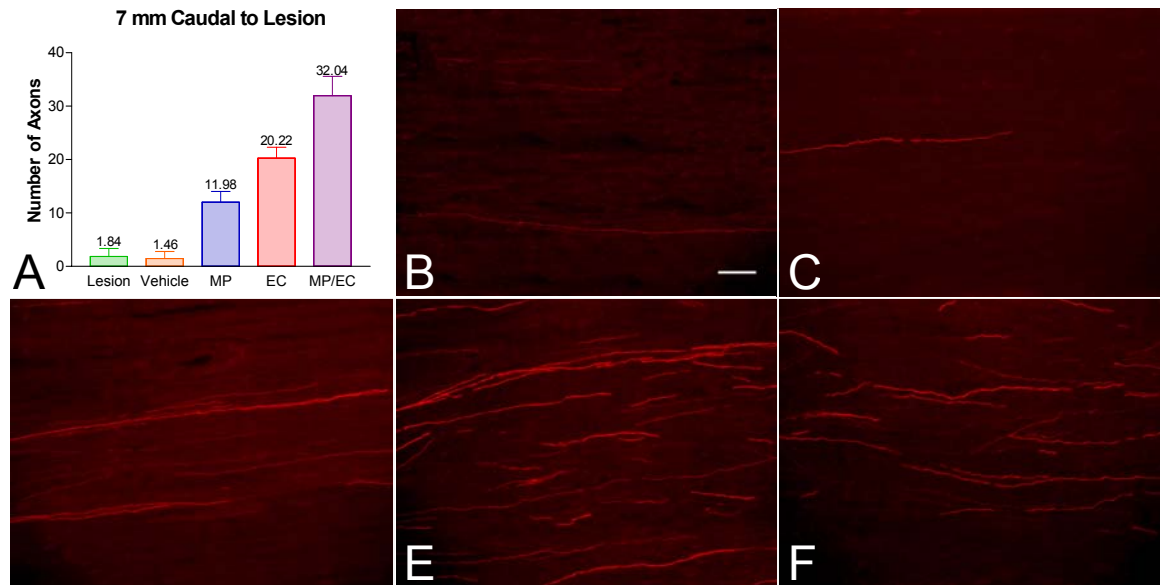


Figure 4: There is no significant difference between the Lesion (B, 1.84 ± 1.51) and Vehicle (C, 1.46 ± 1.32) groups, unlike the MP group (D, 11.98 ± 2.04), which has significantly more axons. The MP group has significantly fewer axons than the EC group (E, 20.22 ± 2.03), and the EC group has significantly fewer axons than the MP/EC group (F, 32.04 ± 3.64).

Figure 5. Data From 13 mm Caudal To The Lesion

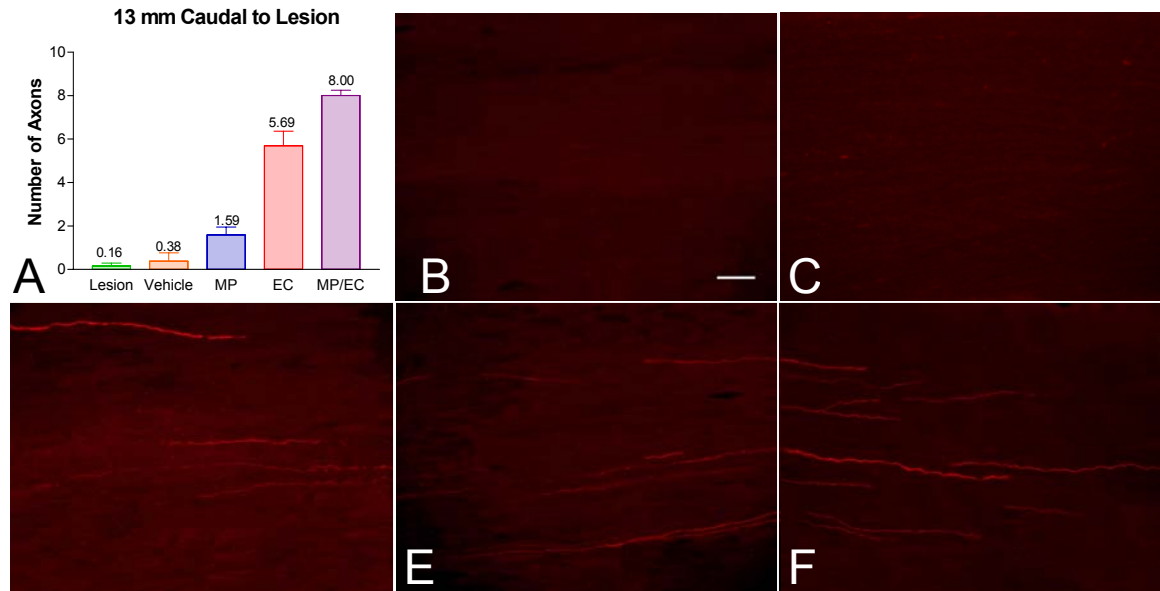


Figure 5: Statistical analysis showed no significant difference between the Lesion (B, 0.16 ± 0.14), Vehicle (C, 0.38 ± 0.38) and MP (D, 1.60 ± 0.36) groups, but that the EC group (E, 5.69 ± 0.68) has significantly more axons than these three groups. There are significantly more axons in the MP/EC group (F, 8.00 ± 0.25) than all other lesioned groups at this distance.

Figure 6. Data From 19 mm Caudal To The Lesion

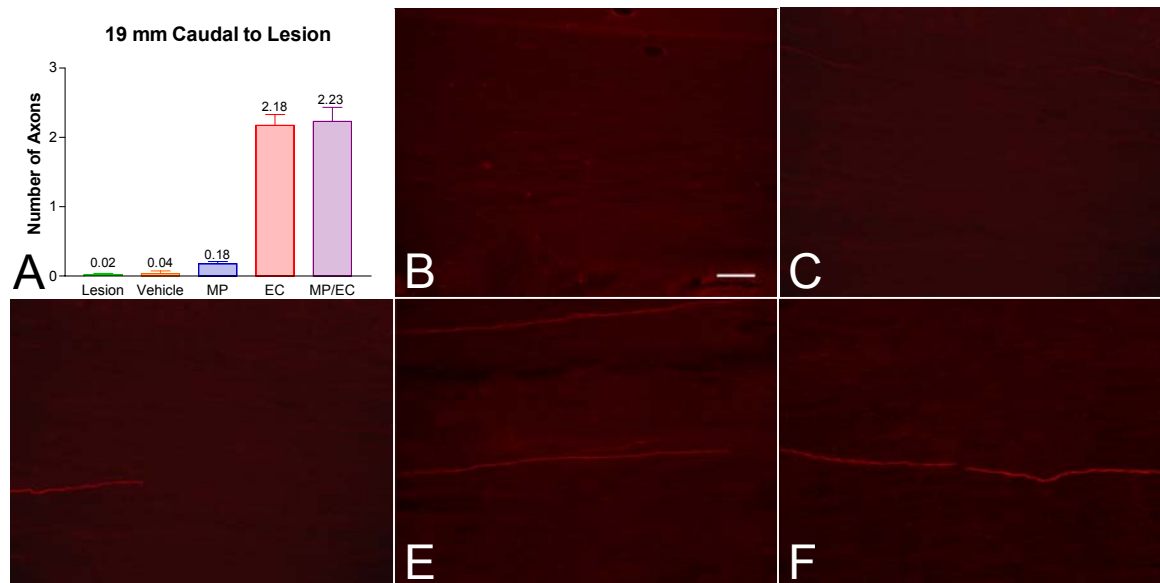


Figure 6: As was found at 13 mm, the Lesion (B, 0.02 ± 0.02), Vehicle (C, 0.04 ± 0.04) groups and MP (D, 0.18 ± 0.03) groups are not statistically different. The EC (E, 2.18 ± 0.16) and MP/EC (F, 2.23 ± 0.20) groups have significantly more axons than all other lesioned groups, but there is no significant difference between these groups.

Figure 7. ECs Co-Localized With Axons

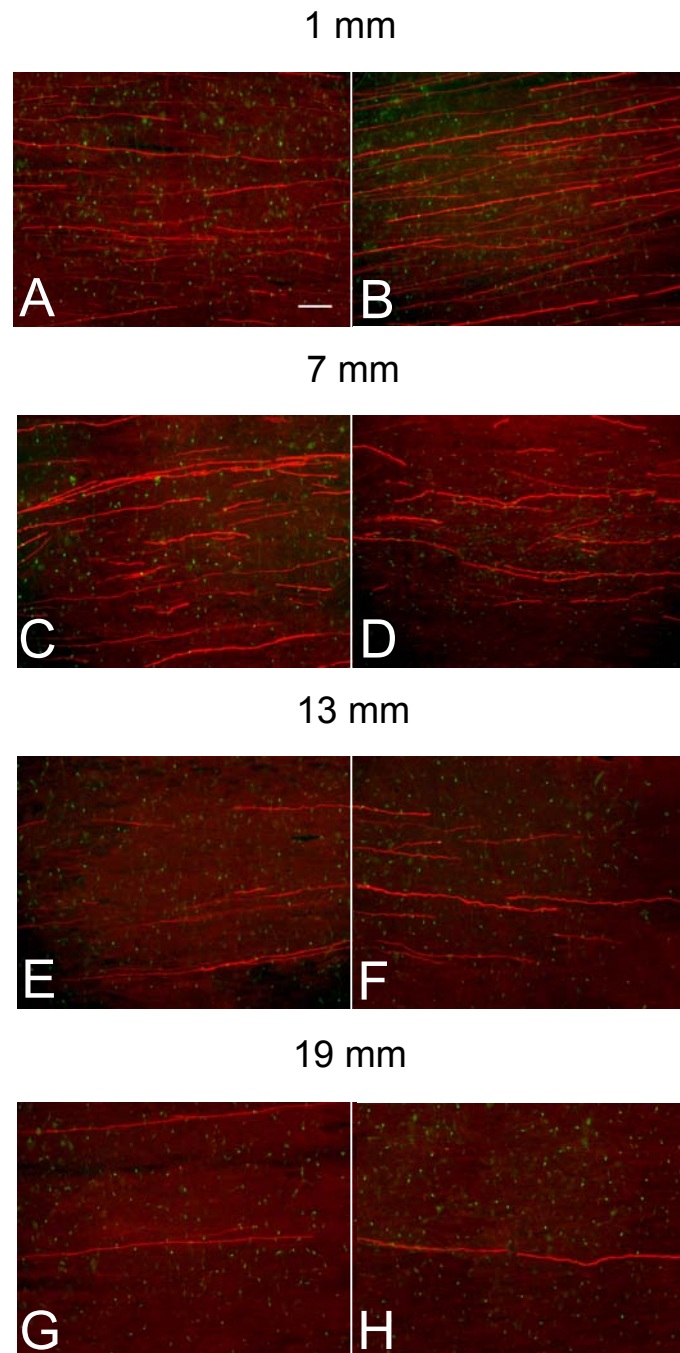


Figure 7: Photomicrographs of BDT-labeled CST axons (shown in red) surrounded by Cell Tracker™ green-labeled ECs. ECs in both the EC and MP/EC groups were at all distances, and representative images are shown from 1 mm (A and B, respectively); 7 mm (C and D), 13 mm (E and F), and 19 mm (G and H), the furthest distance analyzed. Scale bar = 50 μ m.

CHAPTER 5

Discussion

The purpose of this investigation was to determine whether the transplantation of cultured rat ensheathing cells followed by administration of methylprednisolone promotes axonal regeneration and functional reinnervation of CST axons after acute cervical spinal cord injury in adult rats. Three different sets of experiments were performed to complete this investigation. The first set of experiments resulted in a new method for purification of ensheathing cells from the adult rat olfactory bulb. This procedure allowed for the transplantation of purified ensheathing cells into the CST after lesioning. The second set of experiments examined the ability of a modified DFR testing apparatus to assess CST function. These experiments demonstrated that the modified testing apparatus could be used to assess DFR function after bilateral lesioning of the cervical CST, without resulting in extinguished behavior. The final set of experiments explored the ability of ensheathing cells and methylprednisolone to promote morphological and functional repair after SCI. These experiments established that methylprednisolone and purified ensheathing cells, when used in combination, are able to enhance axonal elongation and DFR performance after lesioning of the CST.

The first step necessary to complete this investigation required the purification of ensheathing cells for transplantation into the lesioned spinal cord. Two procedures had previously been reported describing the purification of

ensheathing cells from adult rats. One method resulted in a very low yield of ensheathing cells after purification (Ramon-Cueto and Nieto-Sampedro, 1994). The other method which used immuno-magnetic beads for cell separation resulted in magnetic beads still attached to the ensheathing cells at one month after purification even after multiple washings, medium changes, and transplantation *in vivo* (Gudino-Cabrera and Nieto-Sampedro, 1996).

Therefore, we developed a novel method for purification of ensheathing cells from adult rat olfactory bulbs. The underlying principle for this method of separation is that the cell types harvested from the outer two layers of the adult olfactory bulb have different rates of attachment to various substrates. When seeded onto uncoated slides, fibroblasts (70.4%) settled within one hour and astrocytes (67.6%) attached during the next 24 hours; ensheathing cells remained in the supernatant. Incubation of the supernatant on poly-L-lysine coated slides for 24-48 hours resulted in a large yield of ensheathing cells (1.02×10^6 cell/rat) that was 93% pure (Nash et al., 2001).

The second step towards completion of this investigation was selection of a behavioral test that would accurately assess function of the CST after bilateral, cervical lesioning. While numerous tests exist that examine general motor function after SCI (Rivlin and Tator, 1977; Gale et al., 1985; Kerasidis et al., 1987; Wrathall, 1992a; Basso et al., 1995; von Euler et al., 1996), and hindlimb function after thoracic lesions of the CST (Kunkel-Bagden and Bregman, 1990; Behrmann et al., 1992; Kunkel-Bagden et al., 1992, 1993; Bregman et al., 1995; Palladini et al., 1996; Cheng et al., 1997; Grill et al., 1997; Z'Graggen et al.,

1998), they are not useful for examining forelimb function after CST lesions. While the sticker removal test can be employed to examine forelimb movement (Schrimsher and Reier, 1992; Diener and Bregman, 1998a), it is not sufficient for analyzing varying degrees of CST impairment and improvement, which is reflected in grasping ability. After our modification to the testing apparatus described by Castro (Castro, 1972b), the DFR task was found to be ideal for assessing CST function via examination of grasping ability.

Using the DFR apparatus originally described in the literature (Castro, 1972b), the Sham Control group showed no significant difference between their pre-surgical (95.3%) and post-surgical (93.4%) DFR performance. However, while the Lesioned group performed the DFR task as expected prior to surgery (96.2%), their post-surgical performance was unexpectedly poor (0%). We hypothesized that this poor performance resulted from extinction of the DFR behavior. The apparatus and procedure were modified to prevent the DFR behavior from extinguishing. Using the modified apparatus, testing revealed that, while still impaired post-surgery relative to the Sham Control rats (93.4%), the Lesioned rats were able to perform the DFR task with limited success (39.3%). These data demonstrated that the modified DFR apparatus prevented extinction of the DFR behavior in severely lesioned animals, and could be used to assess CST function after bilateral, cervical lesions. The quantitative data collected from this modified DFR apparatus was indicative of the degree of CST integrity.

The final set of experiments utilized the information obtained from the first two experiments to help address the question of whether transplanted, purified

ensheathing cells, combined with methylprednisolone administration, would result in axonal regeneration and DFR recovery after lesioning of the CST. When administered alone, methylprednisolone promoted axonal regeneration and improved functional recovery after injury when compared to untreated rats. While these improvements were statistically significant, they did not result in complete restoration of DFR function (57%), probably as a result of the limited distance the axons regenerated (~ 7 mm). Treatment with ensheathing cells, whether alone or in combination with methylprednisolone, resulted in axonal elongation to the furthest distance analyzed caudal to the injury site (19 mm) and the highest functional scores of all the lesioned rats. Although more axons were found in the MP/EC group than the EC group at distances up to 13 mm caudal to the lesion, this increase in the number of axons was not enough to result in statistically significant better DFR performance of the MP/EC group (78%) when compared to the EC group (72%). One explanation for this finding is that given a longer recovery period, the larger number of axons seen at shorter distances could extend further, resulting in more functional recovery. Future studies are needed to examine the morphological and functional repair of this combination of therapies after longer recovery periods.

When examining the data from these experiments, it is important to note that when methylprednisolone was used in combination with ensheathing cells, there was not a *decrease* in axonal regeneration or functional recovery. This was a potential concern given that ensheathing cells share phenotypic qualities with astrocytes, which have been shown to act as immune cells (Dong and

Benveniste, 2001), and methylprednisolone is known to suppress the function of immune cells (Wahl et al., 1975; Almawi et al., 1991). Investigation into the mechanisms of action of methylprednisolone and ensheathing cells yielded little insight into how these therapies would interact, since the mechanism for methylprednisolone is still hotly debated and the mechanism for ensheathing cells is just starting to be elucidated. Additional studies examining the mechanisms of actions of ensheathing cells and methylprednisolone on regeneration, both alone and in combination, could be undertaken to further understand how these therapies exert their regenerative effects on the spinal cord.

Many individual therapies have been investigated in the search for a treatment for SCI (Hall et al., 1978; David and Aguayo, 1981; Kuhlengel et al., 1990; Schnell and Schwab, 1993; Ramon-Cueto and Nieto-Sampedro, 1994; Li et al., 1997; Diener and Bregman, 1998a; Houweling et al., 1998). While much progress has been made, a cure is still elusive. Damage to the spinal cord sets many complex processes in motion; the attempted repair of the spinal cord initiates numerous additional events. To repair the spinal cord after injury, future systematic studies are needed to characterize those processes that inhibit regeneration, in order to formulate a comprehensive treatment plan, delivered over the appropriate time course.

Studies have shown that the inflammatory response, initiated during the first few hours after spinal cord injury, contributes to secondary injury (Hirata et al., 1980; Becker and Grasso, 1985; Kontos et al., 1985; Williams and Higgs,

1988; Espersen et al., 1989; Schleimer et al., 1989; Hsu and Dimitrijevic, 1990; Xu et al., 1998), leading to the application of steroids with primarily anti-inflammatory properties as the current first line of defense (Bracken et al., 1984; Bracken et al., 1990; Bracken et al., 1997). If future studies demonstrate that lipid peroxidation of cell membranes also contributes to secondary injury, then a glucocorticoid with anti-lipid peroxidation properties might be administered immediately after injury. If both these mechanisms are found to play critical roles in secondary injury, then a steroid such as methylprednisolone, with both anti-inflammatory and anti-lipid peroxidation properties, could be ideal.

After injury, the CNS attempts to clean the debris out of the damaged regions of the spinal cord (Perry et al., 1987; Dusart and Schwab, 1994). Unlike in the PNS (Bartholdi and Schwab, 1997), clearance of myelin debris after injury is slow in the CNS (Perry et al., 1987; Dusart and Schwab, 1994). CNS myelin has been found to contain molecules that are inhibitory to axonal outgrowth (Caroni et al., 1988; Caroni and Schwab, 1988a, b; Schwab and Caroni, 1988); blocking the inhibitory effects of these molecules may be another line of defense. Previous research has shown that the IN-1 antibody is able to block these inhibitory molecules, resulting in axonal extension (Schnell and Schwab, 1993; Schwab, 1995). It is also possible that cells, such as ensheathing cells, transplanted into the spinal cord may be involved in the destruction of the myelin debris, making the separate application of the IN-1 antibody unnecessary. Future studies that elucidate the mechanisms of action of the cell types shown to promote axonal regeneration could assist in this determination.

Once the problems associated with secondary injury and barriers to regeneration have been rectified, another step future studies could address is the trophic needs of regrowing axons. Motor and sensory axons have been found to require different neurotrophic factors to facilitate regeneration (Bregman et al., 1997a; Oyelese et al., 1997; Houweling et al., 1998). Therefore, future studies might examine what combinations of neurotrophic factors need to be administered to promote the regeneration of these two types of axons. Future studies might also determine, not only the appropriate combination of growth factors, but also the appropriate timing for their application. Future experiments may reveal that different neurotrophic factors need to be applied to different areas of the CNS at different stages of recovery, in order to result in successful repair of the spinal cord.

While different therapies that address each of these problems associated with spinal cord injury could eventually lead to a solution, identification of a few therapies that address all of these problems would be ideal. The transplantation of one or more cell types into the damaged spinal cord may be one of the answers, because cells are capable of performing many functions. A good line of defense in the battle against spinal cord damage may be to identify one or more cell types that are able to halt the “negative” processes that occur after SCI and are also able to nourish the “positive” processes that promote regeneration. Therefore, future studies might characterize the cell types that encourage axonal or functional repair after SCI, to identify which components of SCI each cell type

could resolve, and what additional problems may need to be resolved with supplemental therapies.

One final aspect of SCI that future studies might address is chronic SCI. Most SCI studies have been focused on the acute injury model (Bregman, 1998). However, some studies within the last few years have begun to investigate if neurons have the capacity to regenerate after a chronic injury. Ye and Houle (1997) demonstrated that the application of growth factors to a chronically injured spinal cord (4 weeks post-injury) promoted axonal regrowth. Reticulospinal and rubrospinal axons regrew in response to BDNF and NT-3, while ciliary neurotrophic factor (CNTF) promoted regrowth of axons from most brain regions with a major contribution to descending pathways in the spinal cord. The exception was the motor cortex, with no evidence of axonal regeneration by CST axons. In a subsequent study, Houle and Ye (1997) demonstrated that brain stem spinal neurons exposed to CNTF, at four or eight weeks post-injury, regrew in comparable numbers. However, exposure to basic fibroblast growth factor (bFGF) at eight weeks post-injury decreased regrowth of brain stem spinal neurons by 50% compared to application of bFGF at four weeks. This result indicates that some factors may be effective promoters of regeneration only if provided within a defined post-injury period. Zurita and colleagues (2001) transplanted co-grafts of fetal brain and adult peripheral nerve tissue into the spinal cord 3 months after a crush injury. Eight to twelve months after transplantation, chronically injured rats with transplants were found to have a significant increase in muscle mass in the lower extremities relative to chronically

injured controls. In the rats receiving the transplants, bundles of nerve fibers were found to exit the transplant. In a recent study, Coumans and colleagues (2001) utilized fetal spinal cord transplants and neurotrophins immediately, two weeks, and four weeks after complete spinal cord transection in rats.

Surprisingly, compared to acute treatment, regeneration of supraspinal pathways and recovery of motor function were significantly increased when treatment was delayed until two and four weeks. These studies demonstrate that while some acute therapies may not be applicable after chronic injury, others may actually work better in a chronic model. Clearly, more research into chronic SCI is needed.

While the studies described herein do not solve all the problems associated with SCI, they do provide a clinically relevant step towards reaching a solution. Specifically, these experiments demonstrated that the combined application of methylprednisolone and purified ensheathing cells improve DFR performance and axonal elongation up to 13 mm at six weeks post-injury in rats. Taken together, the findings of this investigation contribute to the progress being made towards treating SCI. A better understanding of these therapies, and all therapies shown to improve outcome after SCI may be essential if a cure for spinal cord injury is to be found.

APPENDIX 1

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November 8, 2001

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